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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Jane J. Zara
Art Unit	:	1635
Applicant	:	William G. Kerr
Serial No.	:	09/955,174
Filed	:	September 19, 2001
For	:	Control of NK Cell Function and Survival by Modulation of SHIP Activity

MS AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF WILLIAM G. KERR, Ph.D., UNDER 37 C.F.R. §1.132

Sir:

I, William G. Kerr, Ph.D., of the University of South Florida, hereby declare:

THAT, my *curriculum vitae* is attached hereto as Exhibit A;

THAT, I am a named inventor on the above-referenced patent application;

THAT, I have read and understood the specification and claims of the subject application and the Office Action dated February 18, 2004;

AND, being thus duly qualified, do further declare:

1. The above-referenced Office Action indicates that claims 1-5, 7-13, 15, and 16 are rejected under 35 U.S.C. §112, first paragraph, as lacking sufficient written description by the patent application.

2. Our invention is based on the unexpected finding that reducing the activity of hematopoietic-specific SH2-containing inositol-5-phosphatase (SHIP) has physiological effects, such as suppression of natural killer (NK) cell-mediated activities, which provide therapeutic benefits in the suppression of transplant rejection and treatment of graft-versus-host disease (GVHD), for example.

3. The Reviewer indicates that the subject patent application does not provide the distinguishing attributes identifying members of "the genus comprising SHIP mRNA". As indicated above, and as recited in the claims of the accompanying Amendment, the subject invention involves the reduction of SHIP function. SHIP (which is also known in the art as SHIP-1, SHIP1, SHIPI, and SHIP-I) was also the subject of Helgason, *et al.* (1998), Huber *et al.* (1998), Liu *et al.* (1999), Liu *et al.* (1998), US. Patent No. 6,090,621 (Kavanaugh *et al.*), PCT publication WO 9710252A1 (Rohrschneider, L.R.), and PCT publication WO 9712039A2 (Krystal, G.), which are cited at pages 2 and 3 of the subject patent application. The methods recited in the claims of the accompanying Amendment involve administering an interfering RNA to a mammal that is specific for SHIP-1 mRNA within the mammal. The mRNA sequences of mouse SHIP-1 and human SHIP-1 have been publicly available since the late 1990s, as evidenced by Exhibits B and C (which are attached hereto), accession numbers NM_10566 and NM_005541, respectively, from the National Center for Biotechnology Information (NCBI) database. Although some nucleotide changes may have been subsequently made to update the GenBank sequences, Exhibits B and C show that the mouse and human SHIP-1 sequences were deposited in GenBank by papers published in 1996 and 1997. Therefore, having the sequence of the target gene, one skilled in the art could readily envision target nucleic acid sequences within the recipient mammal's mRNA. Due to nucleotide complementarity and the mechanism of RNA interference (RNAi), RNA molecules likely to interfere with expression of SHIP-1 could then be determined.

4. The Reviewer indicates that the subject patent application fails to provide particular guidance resolving issues associated with *in vivo* delivery of oligonucleotides and treatment effects. RNAi has been demonstrated to facilitate gene silencing in a variety of animal models including *C. elegans*, zebrafish, and in other biological systems such as *Drosophila* and mammalian cell culture, as reported or described in Zamore *et al.* (*Cell*, 2000, 101:25-33) and Svoboda *et al.* (*Development*, 2000, 127:4147-4156), attached hereto as Exhibit D and E, respectively. As indicated in Exhibits D and E, during the RNAi reaction, both strands of dsRNA are processed to RNA segments 21 to 23 nucleotides in length. The processing of the dsRNA to these fragments does not require the presence

of the targeted mRNA, and the targeted mRNA is cleaved only in the regions of identity to the dsRNA and at sites that are 21 to 23 nucleotides apart (the same interval observed for the dsRNA itself). It was confirmed that the 21 to 23 nucleotide RNA was sufficient to cause sequence specific interference *in vitro* (see page 30, column 2, lines 45-50, of Zamore *et al.*).

5. Exhibit F, which is attached hereto, shows suppression of SHIP-1 expression in embryonic stem (ES) cells *in vitro* by RNAi. ES cells that express the SHIP-1 gene were transfected with an irrelevant shRNA vector (Lane 3) or with two different shRNA vectors that produced siRNAS specific for SHIP-1 (Lanes 4 and 5). The cells were then lysed and equal quantities of whole cell extracts were immuno-blotted with either anti-SHIP-1 (Panel A) or anti-Actin (Panel B). Lane 2 shows untreated ES cells. Lane 1 shows untreated RAW264.7 mouse myeloid cells that express the SH2-containing SHIP-1 p135 and p145 isoforms. Panel A shows significant reduction of SHIP expression in primary ES cells after transfection of SHIP-1-specific shRNA vectors in the absence of selection. It would be expected that these vectors would also interfere with expression of the larger SH2-containing isoforms in differentiated hematopoietic cells.

6. Exhibit G, which is attached hereto, shows induction of SHIP-1 deficiency *in vivo* by RNAi increases the frequency of circulating myeloid cells including cells with a myeloid suppressor cell phenotype. In this experiment, it was confirmed that RNAi can effectively knockdown SHIP-1 expression *in vivo* using techniques described in the subject patent application. Two mice were injected with a SHIP-1 shRNA vector complexed with the cationic lipid 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP) while two additional mice received an irrelevant shRNA vector specific for the human LRBA gene. The design and sequence of the shRNA vector is shown in Exhibit H, which is attached hereto. The mice that received the SHIP-1-specific shRNA vector showed significant suppression of all major SHIP isoforms in the spleen, while β-actin levels were essentially unaltered, as shown in Figure A of Exhibit G. We also screened four different SHIP-1 specific siRNAs for knockdown of SHIP-1 in the RAW264.7 mouse myeloid cell line or ES cells. The two best siRNAs, #1 and #4, were pooled and tested *in vivo*. SiRNAs #1 and #4 were pooled,

complexed with DOTAP and injected intravenously into two separate mice. Two additional mice received the same mass of irrelevant GL2 siRNA control. As with SHIP-1 shRNA-treated mice, there was partial suppression of SHIP-1 expression in peripheral blood mononuclear cells (PBMC) by Western blotting 20 hours after the treatment (data not shown). We also examined the impact on the myeloid compartment in PBMC and found a significant increase in Mac+Gr1-monocytes and circulating Mac1+GR1+ cells (myeloid suppressor cells) in the SHIP-1 siRNA treated mice, relative to the GL2 control animals, as shown in Figure B of Exhibit G. The sequences of siRNAs #1-4 and their respective target sites within the open reading frame of mouse SHIP-1 are shown in Exhibit I, which is attached hereto. These findings show that knockdown of SHIP-1 expression *in vivo* by RNAi is a feasible approach that can exert physiological effect even with partial knockdown of SHIP-1 expression.

7. As indicated at page 11, lines 10-34, and page 12, lines 1-8, of the subject patent application, polycationic molecules such as liposomes can be used as gene delivery vehicles to deliver genetic constructs for reduction of SHIP expression. Cationic liposomes such as DOTAP are positively charged and interact with the negatively charged DNA molecules to form a stable positively charged DNA/liposome complex that binds to the negatively charged surface of the cell, where it is internalized. Column 17 of U.S. Patent No. 6,025,198, which is cited by the Reviewer in the Office Action, indicates that cationic liposomes may be used to deliver antisense oligonucleotides to inhibit expression of SHIP-2. DOTAP has been used for transfection of mammalian cells *in vitro* and *in vivo* for some time (see, for example, Porteous D.J. *et al.*, "Evidence for safety and efficacy of DOTAP cationic liposome mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis", *Gene Ther.*, 1997, Mar., 4(3):210-218; Song Y.K. *et al.*, "Characterization of cationic liposome-mediated gene transfer *in vivo* by intravenous administration", *Hum. Gene Ther.*, 1997, Sept., 8(13):1585-1594). However, in addition to such non-viral delivery vehicles, viral delivery vehicles such as adenovirus and adeno-associated virus could also be utilized to deliver interfering RNA to reduce SHIP-1 expression, as taught at pages 12 and 13 of the subject patent application.

8. Based on the experimental data demonstrating the ability to reduce expression of SHIP-1 *in vivo* in accordance with the teaching of the subject patent application, and the observed effects of SHIP-1 deficiency on NK cell function and GVHD in SHIP-/- transgenic mice (Examples 2-6 of the subject patent application), there is no reason to doubt that reduction of SHIP-1 function by RNA interference or other means of SHIP-1 inhibition will be of therapeutic benefit in suppressing transplant rejection and graft-versus-host disease in mammals, including humans.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed: William G. Kerr

William G. Kerr, Ph.D.

Date: 7/16/04

Curriculum Vitae
William G. Kerr

Date: May 6, 2003

Home Address: 4421 W. Watrous Ave.
Tampa, FL 33629

Office Address: SRB2
Immunology Program
Moffitt Cancer Center
University of South Florida
12902 Magnolia Dr.
Tampa, FL 33612

Education:
1978-1982 B.S. Lehigh University (Chemistry/Molecular Biophysics)
1982-1987 Ph.D. University of Alabama at Birmingham (Molecular and Cellular Biology)

Postgraduate Training and Fellowship Appointments:
1987-1993 Post-doctoral Fellow
Department of Genetics
Stanford University

Industrial Employment:
1993-1995 Research Group Leader,
Cancer Gene Therapy, SyStemix, Inc.
Palo Alto, CA

1996 Visiting Scientist
DNAX Research Institute
Palo Alto, CA

Academic Appointments:
1996-2000 Assistant Professor
Dept. of Molecular and Cellular Engineering
University of Pennsylvania School of Medicine
Philadelphia, PA

2000-present Associate Professor (with tenure)
Dept. of Interdisciplinary Oncology
H. Lee Moffitt Comprehensive Cancer Center
University of South Florida
Tampa, FL

2000-present Associate Professor
Dept. of Biochemistry and Molecular Biology
H. Lee Moffitt Comprehensive Cancer Center
University of South Florida
Tampa, FL

2000–2001 Vice Director
Institute for Biomolecular Sciences
University of South Florida

Awards, Honors and Membership in Honorary Societies:

1981 Dean's List - Lehigh University
1981-1982 Bethlehem Fabricators Scholarship - Lehigh University
1986-1987 Pre-Doctoral Fellow - NIH (UAB)
1987 First Place - Sigma Xi Graduate Research Competition (UAB)
1987-1989 Post-Doctoral Fellow - NIH (Stanford Univ.)
1990-1993 Fellow - Irvington Institute for Medical Research (Stanford Univ.)
1997 McCabe Fund (University of Pennsylvania)
2002-2007 The Newman Scholar of the Leukemia and Lymphoma Society
2003 Outstanding Faculty Research Achievement Award (University of South Florida)
2004-present Full Member, Sigma Xi

Other Activities:

Member, Massachusetts Breast Cancer Research Review Panel (1997-2000)
Member, Univ. of California Breast Cancer Research Program (2000-2003)
Member, Special Emphasis Review Panel - Novel HIV Therapies: Integrated Preclinical/Clinical Program, NIAID/NIH (1999)
Member, Special Emphasis Review Panel – T/NK Cell Immunity, NIAID/NIH (2003-2004)
Member, Biomedical Resource Review Panel/NIH, P41 National Resource for Imaging Mass Spectrometry (1999-2000)
Ad Hoc Reviewer, VA Intramural Grants Program (1998-1999)
Ad Hoc Reviewer, Israel Science Foundation (1998)
Ad Hoc Reviewer, *Human Gene Therapy, Gene Therapy, Cytometry, Leukemia, J. Immunotherapy. J. Immunology, BLOOD, Gene, Proc. Natl. Acad. Sciences*
Member, American Association of Immunologists (1999 – present)
Active Member, American Society of Hematology (2001– present)
Member, International Society for Stem Cell Research (2003-present)
Consultant, Genentech (1998)
Consultant, Sunkyong Group (2000)
Consultant, Saneron Ccell (2003-2005)

Publications:

1. **Kerr, W.G.**, Cooper, M.D., Feng, L., Burrows, P.D., Hendershot, L.M. Mu heavy chains can associate with a pseudo-light chain complex (ΨL) in human pre-B cell lines. *International Immunology* 1: 355-361, 1989.

2. **Kerr, W.G.**, Nolan, G.P., Herzenberg, L.A. *In situ* detection of transcriptionally-active chromatin and genetic regulatory elements in individual viable mammalian cells. *Immunology* 68[Suppl. 2]: 74-79, 1989.

3. **Kerr, W. G.**, Nolan, G.P., Serafini, A.T., Herzenberg, L.A. : Transcriptionally-defective retroviruses containing *lacZ* for the *in situ* detection of endogenous genes and developmentally-regulated chromatin. *Cold Spring Harbor Symposium on Quantitative Biology* 54: 767-776, 1989.

4. Burrows, P.D., Kubagawa, H., Nishimoto, N., **Kerr, W.G.**, Borzillo, G.V., Hendershot, L.M., Cooper, M.D. Differences in human B cell differentiation. *Adv. Exp. Biol. Med.* 292: 215-226, 1991.

5. **Kerr, W.G.**, Herzenberg, L.A. Gene-search viruses and FACS-Gal permits the detection, isolation and characterization of mammalian cells with *in situ* fusions between cellular genes and *E. coli lacZ. Methods: A Companion to Methods in Enzymology* 2: 261-271, 1991.

6. **Kerr, W.G.**, Hendershot, L.M., Burrows, P.D. Regulation of IgM and IgD expression in human B-lineage cells. *J. Immunol.* 146: 3314-3321, 1991.

7. **Kerr, W.G.**, Nolan, G.P., Johnsen, J., Herzenberg, L.A. *In situ* detection of stage-specific genes and enhancers in B cell differentiation via gene-search retroviruses. *Adv. Exp. Biol. Med.* 292: 187-200, 1991.

8. **Kerr, W.G.**, Burrows, P.D. Stage-specific transcription of germline IgH Cγ and Cα regions during human B cell differentiation. *International Immunology* 3: 1059-1065, 1991.

9. **Kerr, W.G.** and Mulé, J.J. Gene therapy: Current status and future prospects. *J. Leuko. Biol.* 56: 210-214, 1994.

10. Gerard, C.J., Arboleda, M.J., Solar, G., Mulé, J.J., **Kerr, W.G.** A rapid and quantitative assay to estimate gene transfer into retrovirally-transduced hematopoietic stem/progenitor cells using a 96-well format PCR and fluorescent detection system universal for MMLV-based proviruses. *Human Gene Therapy* 7: 343-354, 1996.

11. **Kerr, W.G.** Progress towards a new wave of immune-based therapeutics. *Trends in Biotechnology*, 14: 359-360, 1996.

12. **Kerr, W.G.**, Heller, M.R., Herzenberg, L.A. Analysis of LPS-response genes in B-lineage cells demonstrates that they can have differentiation stage-restricted expression and contain SH2 domains. *Proc. Natl. Acad. Sci. (USA)* 93, 3947-3952, 1996.

13. Zambrowicz, B.P., Imamoto, A., Fiering, S., Herzenberg, L.A., **Kerr, W.G.**, Soriano, P. Disruption of overlapping transcripts in the ROSA βgeo26 gene trap strain leads to widespread expression of βgalactosidase in mouse embryos and hematopoietic cells. *Proc. Natl. Acad. Sci. (USA)* 94, 3789-3794, 1997.

14. Lorincz, M., Herzenberg, L.A., Diwu, Z., Barranger, J.A., and **Kerr, W.G.** Detection and isolation of gene-corrected cells in Gaucher disease via a FACS assay for lysosomal glucocerebrosidase activity. *BLOOD* 89, 3412-3420, 1997.

15. Kapasi, Z.F., Quin, D., **Kerr, W.G.**, Kosko-Vilbois, M.H., Schultz, L., Tew, J.G., and Szakal, A.K. Follicular dendritic cell (FDC) precursors in primary lymphoid tissues. *J. Immunol.* 160, 1078-1084, 1998.

16. Solar, G., **Kerr, W.G.**, Ziegler, F., Hess, D., Donahue, C., DeSavauge, F.J., and Eaton, D. *Special Focus:* Role of *c-mpl* in early hematopoiesis. *BLOOD* 92, 4-10, 1998.

17. **Kerr, W.G.** Genetic modification of the hematolymphoid compartment for therapeutic purposes. *Hematology/Oncology Clinics of North America: Gene Therapy* 12: 503-518, 1998.

18. Spain, L. and **W.G Kerr.** Lymphoid development. ENCYCLOPEDIA OF LIFE SCIENCES (Macmillan Reference Ltd), 1999.

19. Greenberg, A.W., ***Kerr, W.G.**, *Hammer, D.A. Relationship between selectin-mediated rolling of hematopoietic stem/progenitor cells and progression in hematopoietic development. *BLOOD* 95, 478-486, 2000. (**Senior authorship is shared on this manuscript*)

20. Wang JW, Howson J, Ghansah T, Ninos J, Kerr WG. Inhibition of apoptosis by the BEACH domain and WD repeats of gene lba that has key features of both protein kinase A anchor and chs1/beige genes. Scientific World Journal 1(1 Suppl. 3), 96, 2001.

21. Wang, J.W., Howson, J., Haller, E. and **Kerr, W.G.** Identification of a novel LPS-inducible gene that has key features of both protein kinase A anchor and CHS1/BEIGE proteins. *Journal of Immunology* 166, 4586-4595, 2001.

22. Ghansah, T., Ninos, J. and **Kerr, W.G.** A role for the SH2-containing Inositol Phosphatase (SHIP) in the biology of stem cells and natural killer cells. In *Activating and Inhibitory Immunoglobulin-Like Receptors (Eds, M.D. Cooper, T. Takai and J.V. Ravetch, Springer-Verlag),* pp. 129-140, 2001.

23. Zheng Tu, John M. Ninos, Zhengyu Ma, Jia-Wang Wang, Maria P. Lemos, Caroline Desponts, Tomar Ghansah, Julie M. Howson and **Kerr, W.G.** *Plenary Paper:* Embryonic and hematopoietic stem cells express a novel SHIP isoform that partners with the Grb2 adapter protein. *BLOOD* 98, 2028-2038, 2001.

24. **Kerr, W.G.**, Eaton, D.L. and Solar, G.P. Monoclonal antibodies specific for *c-mpl* for the study of human megakaryopoiesis and stem cell biology. In: *Leukocyte Typing VII,* Ed. D. Mason, Oxford University Press, UK, pp.561-563, 2002.

25. Wang, J.W., Howson, J.M. Ghansah, T., Desponts, C., Ninos, J.M., May, S.L., Nguyen, K.H.T., Sorimachi, N. and **Kerr, W.G.** Influence of SHIP on the NK repertoire and allogeneic bone marrow transplantation. *SCIENCE* 295, 2094-2097, 2002.

26. Cheng, F., Wang, H., Cuenca, A., Huang, M. Ghansah, T., Brayer, J., **Kerr, W.**, Takeda, K., Akira, S., Schoenberger, S., Yu, H., Jove, R. and E. Sotomayor. Critical role for Stat3 signaling in immune tolerance. *Immunity* 19, 425-436, 2003.

27. Wang, J.W., Gamsby, J., Bloom, G., Yeatman, T., Chodosh, L., Cress, W.D., Chen, J. and **Kerr, W.G.** Deregulated expression of LRBA facilitates cancer cell growth. *Oncogene* 23, 4089-4097, 2004.

28. Eason, D.D., Litman, R.T., Luer, C.A., **Kerr, W.G.** and Litman, G.W. Expression of Individual immunoglobulin genes occurs in an unusual system consisting of multiple

independent loci. *European Journal of Immunology*, in press (2004).

29. Perez, L., Desponts, C, Parquet, N and Kerr, W.G. A role for SHIP-1 in the control of megakaryocytopoiesis. *Submitted.*

30. Ninos, J.M., Eaton, D., and **Kerr, W.G.** The TPO receptor, *c-mpl*, demarcates human hematopoietic stem cells from multiple sources and is expressed by cells capable of multi-lineage repopulation following serial transfer. *Submitted.*

31. Ghansah, T., Nguyen, K.H.T., Highfill, S., Desponts, C., May, S., McIntosh, J.K., Brayer, J., Cheng, F., Sotomayor, E. and **Kerr, W.G.** Expansion of myeloid suppressor cells in $SHIP^{-/-}$ mice represses allogeneic T cell responses. *Submitted and under revision.*

32. Hess, D., Brown-Whitehorn, T, Howson, J., Ford, B., McIntosh, H. and **Kerr, W.G.** Repopulation of human lymph node grafts and long-term IgG production in immunodeficient mice co-transplanted with primary and secondary human lymphoid tissue. *Submitted and under revision.*

Current Extramural Grant Support:

1999-2004 PO1 NS27405: *In Vivo* Model of Human Microglia Development and Infection (Project Leader)

2002-2006 RO1 HL72523: Role of SHIP in the Control of NK Cell Function (Principal Investigator)

2002-2007 Scholar Award, Leukemia and Lymphoma Society of America (PI)

2004-2006 Antibody Therapeutics in Multiple Myeloma, Genentech (PI)

Lectures by Invitation:

September, 1994 "Retroviral Transduction of $CD34^{+}Thy^{+}Lin^{-}$ Hematopoietic Stem Cells From Adult Mobilized Peripheral Blood" - Gene Therapy Conference, Cold Spring Harbor, NY

December, 1994 "Retroviral Transduction of $CD34^{+}Thy^{+}Lin^{-}$ Hematopoietic Stem Cells From Adult Mobilized Peripheral Blood" - Annual Meeting of the American Society of Hematology, Nasheville, TN

December, 1994 "Retroviral Transduction of $CD34^{+}Thy^{+}Lin^{-}$ Hematopoietic Stem Cells From Adult Mobilized Peripheral Blood" - Third International Conference on Gene Therapy for Inherited Deficiencies and Disease, London, UK

July, 1995 "High-Efficiency Transduction of Highly-Purified $CD34^{+}Thy^{+}Lin^{-}$ Human Peripheral Blood Hematopoietic Stem Cells with Pseudotyped Retroviruses" - International Congress of Immunology, San Francisco, CA

September 29, 1995 "Gene Transfer Into Highly Purified Human Stem Cells Using Amphotropic and Pseudotyped MMLV-Based Vectors"

- First Conference on Hematopoietic Stem Cell Gene Therapy: Biology and Technology, Bethesda, MD

November, 1995 "High-Efficiency Transduction of Highly-Purified $CD34^{+}Thy^{+}Lin^{-}$ Human Peripheral Blood Hematopoietic Stem Cells with Pseudotyped Retroviruses" - Fourth International Conference on Gene Therapy of Cancer, San Diego, CA

December, 1995 "Directed Immunity" - Immunotherapeutic Strategies for Cancer: Novel Vaccine Strategies, San Diego, CA

March, 1996 "Gene Transfer Into Human Hematopoietic Stem/Progenitor Cells and Potential Therapeutic Applications" - Institute for Genetics, University of Cologne, Cologne, Germany

March, 1996 "Gene Transfer Into Human Hematopoietic Stem/Progenitor Cells and Potential Therapeutic Applications" - Max Planck Institute for Immunobiology, Freiburg, Germany

May, 1996 "Gene Transfer Into Human Hematopoietic Stem/Progenitor Cells and Potential Therapeutic Applications" - Genomic Sciences Series Conference on Gene Therapy, Hilton Head, SC

June, 1996 "Directing Immunity to Cancer Cells via HSC-Based Gene Therapy" - Antigen Processing and Presentation: Novel Therapeutics Development, Bethesda, MD

November, 1996 "Hematopoietic Stem Cell Based Gene Therapies for Cancer: Potential Therapeutic Approaches and Technologies" - Immunotherapeutic Strategies for Cancer, San Diego, CA (Chairman - Session on *Hematopoietic Stem Cell-Based Therapies*)

March 27, 1997 "Hematopoietic Stem Cell Gene Therapy: Will It Cure What Ails Us?", Genentech, South San Francisco, CA

April 23, 1997 "Progress Toward Stem Cell Gene Therapy", Laboratory of Tumor Biology and Immunology, National Cancer Institute, Bethesda, MD

May 9, 1997 "Progress Toward Stem Cell Gene Therapy and SCID-*hu* Models to Assist Us", Symposium on Nonhuman Primate Gene Therapy, New Orleans, LA

February 25, 1998 "*In Vivo* Models of the Human Lymph Node to Study Function and Pathogenesis in the Human Immune System", Immunotherapeutic Strategies for Cancer: Moving Towards the Clinic, San Diego, CA.

June 2, 1998 "Role of *c-mpl* in the Biology of Hematopoietic Stem Cells in Mouse and Man", Center for Molecular Pathogenesis, Umea University, Umea, Sweden.

January 21, 1999 "Modeling the Human Hematolymphoid System in Mice", Penn State Medical College, Hershey, PA.

March 18, 1999 "Role of *c-mpl* in the Hematopoietic Stem Cell Biology", Dept. of Pathology and Lab Medicine, University of Pennsylvania.

March 23, 1999 "Modeling the Human Hematolymphoid System in Immunodeficient Mice", Genzyme Corp., Cambridge, MA.

March 29, 1999 "Modeling the Human Hematolymphoid System in Immunodeficient Mice", Genentech, Inc., South San Francisco, CA.

May 18, 1999 "Studying the Function of the Human Hematopoietic Stem Cell and the Lymphoid System in Immunodeficient Mice", Philadelphia City-Wide AIDS Symposium, Jefferson Medical College, Philadelphia, PA

August 16, 1999 "Role of *c-mpl* in the Hematopoietic Stem Cell Biology", Immunotherapeutic Approaches to Cancer, San Diego, CA.

June 19-24, 2000 "Monoclonal antibodies specific for human *c-mpl* for clustering and study of hematopoiesis", Platelet Workshop - 7th Workshop and Conference on Human Leucocyte Differentiation Antigens, Harrogate, United Kingdom

September 19-20, 2000 "The SH2-Containing Inositol Phosphatase (SHIP) is a crucial regulator of NK cell repertoire and function.", The CREST International Symposium on Immunoglobulin-Like Receptors, Sendai, Japan

October 11-15, 2000 "The SH2-Containing Inositol Phosphatase (SHIP) is a crucial regulator of NK cell repertoire and function", Aegean Conference On Innate Immunity, Santorini, Greece.

October 27, 2000 "Key Genetic Determinants of Stem Cell Biology and Transplantation", Hematopoiesis and Immunology Seminar Series, Johns Hopkins University School of Medicine

April 13, 2001 "The SH2-Containing Inositol Phosphatase (SHIP) is a crucial regulator of NK cell repertoire and function", NCI/Frederick, Frederick, Maryland

April 27, 2001 "The SH2-Containing Inositol Phosphatase (SHIP) is a crucial regulator of NK cell repertoire and function", Dept. of Microbiology and Immunology, Univ. of California San Francisco

July 25, 2001 "SHIP is Critical for Repertoire Diversity and Function in the Adult NK Cell Compartment", Workshop: The role of NK and NKT cells in immune effector mechanisms, 11th International Congress of Immunology, Stockholm, Sweden.

September 4, 2001 "Critical role for SHIP in engraftment of histo-incompatible stem cells", Millenium International Conference: Stem Cell Differentiation, Genetic Reprogramming and Programmed Cell Death, Santorini, Greece.

March 20, 2002 "SHIP Influences the NK Repertoire and Allogeneic Bone Marrow Transplantation", Keystone Symposium: Molecular and Cellular Biology of Leukocyte Regulatory Receptors, Tahoe City, California

April 9, 2002 "Role for SHIP in Stem Cell Biology and Transplantation" Genentech, South San Francisco, California

October 13, 2002 "A Role for SHIP in Allogeneic Bone Marrow Transplantation", Molecular Targets for Cancer Therapy: 2nd Biennial Meeting, St. Petersburg, FL

November 12, 2002, "Role for SHIP in Stem Cell Biology and Transplantation", Molecular and Cellular Biology Seminar Series, UAB, Birmingham, Alabama

January 6, 2003 "A Role for SHIP in Allogeneic Bone Marrow Transplantation" Suntory Pharmaceuticals Research Laboratory, Cambridge, MA

January 14, 2003 "SHIP and LRBA: Two Novel Targets in Cancer Therapy?", Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

June 13, 2003 "Role for SHIP in Stem Cell Biology and Transplantation" Immunology Seminar Series, National Cancer Institute, Frederick, MD

September 7-9, 2003 Session Chair, Lymphopoiesis II: 70th Birthday Symposium for Max Cooper, M.D. UAB, Birmingham, AL

September 19, 2003 "Role for SHIP in Stem Cell Biology and Transplantation", Dept. of Biology Seminar Series, University of North Florida, Jacksonville, Florida (*invited*)

March 21-23, 2004 "A Role for SHIP in Stem Cell Biology and Transplantation", Session: Stem Cells and Cancer (Chair), *Emerging Cancer Treatment Modalities: From Research to Practice*, Copper Mountain, Colorado

April 29, 2004 "A Role for SHIP in Stem Cell Biology and Bone Marrow Transplantation", University Hospital Utrecht, The Netherlands

May 4, 2004 "A Role for SHIP in Stem Cell Biology and Bone Marrow Transplantation", Central Laboratory for the Blood (CLB/Sanquin), Amsterdam, The Netherlands

June 10, 2004 "A Role for SHIP in Stem Cell Biology and Transplantation",

University of California at San Francisco, San Francisco, CA (*Invited*)

June 22, 2004 "A Role for SHIP in Stem Cell Biology and Bone Marrow Transplantation", Amgen, Thousand Oaks, CA (*Invited*)

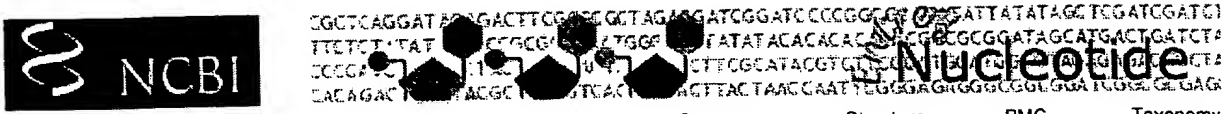
Teaching Responsibilities at the University of Pennsylvania (1996-1999):

Member, Cellular and Molecular Biology (CAMB) Graduate Group (1996-1999)
Member, Immunology Graduate Group (1997-1999)
Chair, Thesis Committee for Steven Suter, Ph.D., Candidate in Cell and Molecular Biology
Member, Thesis Committee for Adam Greenberg, Ph.D. Candidate in Bioengineering
Committee Member, Preliminary Exam of Xiarong Wang, Ph.D. candidate Cell and Molecular Biology Graduate Group
Committee Member, Preliminary Exam of Matthew McLeod, Ph.D. candidate Cell and Molecular Biology Graduate Group (1999)
Mentor, Second Preliminary Exam of Fang Zhao, Ph.D. candidate Immunology Graduate Group (1998)
Mentor, Second Preliminary Exam of Zhengyu Ma, Ph.D. candidate Immunology Graduate Group (1999)
Thesis Advisor, Zheng Tu, Ph.D. candidate, Cell and Molecular Biology Graduate Group (1998)
CAMB 610: Molecular Basis of Gene Therapy (1996)
Lecture 1: The hematopoietic stem cell
Lecture 2: Adenosine deaminase deficiency
CAMB 610: Molecular Basis of Gene Therapy (1997-1999)
Lecture 1: Hematopoiesis
Lecture 2: Discussion session
CAMB 633: Advanced Seminar in Cancer Gene Therapy (Co-Director) (1997)
Lecture 1: Immunotherapy I: Cytokine stimulated tumor immunity
Lecture 2: Immunotherapy II: Genetic modification to directly alter effector function
Lecture 3: Immunotherapy IV: Second generation tumor vaccine approaches
Lecture 4: Selective infection and induction of apoptosis in tumore cells to purge bone marrow
CAMB 633: Advanced Seminar in Gene Therapy (1997)
Lecture: Gaucher Disease
Immunology 660: Developmental Immunology (1996)
Lecture: The Hematopoietic Stem Cell
Immunology 506: Immune Mechanisms (1997-98)
Lecture: Acquired Immunity
Immunology 506: Immune Mechanisms (1998-1999)
Lecture: Role of the Antigen Receptor in B Cell Biology
Immunology 508: Immune Responses (1997-1999)
Lecture: Gene therapy approaches to correcting immunodeficiency states
CAMB 633: Advanced Seminar in Gene Therapy (1998)
Lecture: Modeling the human immune system in immunodeficient mice

CAMB 605: CAMB First Year Ph.D. Student Seminar (Fall 1998)

Teaching and Service at the University of South Florida (2000-present):
Lecturer, Biochemistry 6806: Biochemical Signal Transduction
Lecture: Inositol Phospholipid Signaling (Spring 2000)
Course Director, Stem Cell Biology (Spring 2001)
Course Director, GMS 6055: Immunology and Immunotherapy (2002,2004)
Member, Cancer Biology Education Committee (2000 - present)
Chair, Cancer Biology Graduate Admissions Committee (2001 - 2002)
Member, Biochemistry Graduate Admissions Committee

Lecturer, GMS 6055: Immunology and Applied Cancer Biology (2002-3)
Chair, Thesis Committee for Deborah Kuhn (Ph.D. Candidate in Cancer Biology)
Ph.D. Thesis Advisor, Caroline Desponts (Biochemistry/IBS), Joshua Gamsby (Biochemistry), Joseph Wahle (Cancer Biology)
Mentor, Acquanetta Henry, Undergraduate Honors Student/USF McNair Scholar
Chair, Thesis Committee for Deborah Kuhn (Ph.D. Candidate in Cancer Biology)
Organizer, Moffitt Cancer Center Immunology Colloquium (2002-2004)
Member, Organizing Committee for the Moffitt Cancer Center Molecular Targets Symposium, St. Petersburg, FL (2002)



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ACCESSION NM_010566
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REFERENCE 1 (bases 1 to 4865)
AUTHORS Giuriato,S., Pesesse,X., Bodin,S., Sasaki,T., Viala,C., Marion,E., Penninger,J., Schurmans,S., Erneux,C. and Payrastre,B.
TITLE SH2-containing inositol 5-phosphatases 1 and 2 in blood platelets: their interactions and roles in the control of phosphatidylinositol 3,4,5-trisphosphate levels
JOURNAL Biochem. J. 376 (Pt 1), 199-207 (2003)
PUBMED 12885297
REMARK GeneRIF: SHIP1 plays a major role in controlling phosphatidylinositol 3,4,5-trisphosphate levels in platelets.

REFERENCE 2 (bases 1 to 4865)
AUTHORS Jiang,X., Stuible,M., Chalandon,Y., Li,A., Chan,W.Y., Eisterer,W., Krystal,G., Eaves,A. and Eaves,C.
TITLE Evidence for a positive role of SHIP in the BCR-ABL-mediated transformation of primitive murine hematopoietic cells and in human chronic myeloid leukemia
JOURNAL Blood 102 (8), 2976-2984 (2003)
PUBMED 12829595
REMARK GeneRIF: SHIP expression appears to be differently altered in the early and late stages of differentiation of BCR-ABL-transformed cells

REFERENCE 3 (bases 1 to 4865)
AUTHORS Baran,C.P., Tridandapani,S., Helgason,C.D., Humphries,R.K., Krystal,G. and Marsh,C.B.
TITLE The inositol 5'-phosphatase SHIP-1 and the Src kinase Lyn negatively regulate macrophage colony-stimulating factor-induced Akt activity
JOURNAL J. Biol. Chem. 278 (40), 38628-38636 (2003)
PUBMED 12882960
REMARK GeneRIF: SHIP-1 and Lyn have roles in the negative regulation of M-CSF-R-induced Akt activation

REFERENCE 4 (bases 1 to 4865)
AUTHORS Moody,J.L., Pereira,C.G., Magil,A., Fritzler,M.J. and Jirik,F.R.
TITLE Loss of a single allele of SHIP exacerbates the immunopathology of Pten heterozygous mice
JOURNAL Genes Immun. 4 (1), 60-66 (2003)
PUBMED 12595903
REMARK GeneRIF: Reduced expression of SHIP, achieved by generating mice doubly heterozygous for Pten and SHIP, leads to a worsening of the Pten+/-phenotype.

REFERENCE 5 (bases 1 to 4865)
AUTHORS Nakamura,K., Malykhin,A. and Coggeshall,K.M.
TITLE The Src homology 2 domain-containing inositol 5-phosphatase negatively regulates Fcgamma receptor-mediated phagocytosis through immunoreceptor tyrosine-based activation motif-bearing phagocytic receptors
JOURNAL Blood 100 (9), 3374-3382 (2002)
PUBMED 12384440
REMARK GeneRIF: SHIP negatively regulates FcgammaR-mediated phagocytosis through all ITAM-containing IgG receptors using a molecular mechanism distinct from that in B cells.
REFERENCE 6 (bases 1 to 4865)
AUTHORS Takeshita,S., Namba,N., Zhao,J.J., Jiang,Y., Genant,H.K., Silva,M.J., Brodt,M.D., Helgason,C.D., Kalesnikoff,J., Rauh,M.J., Humphries,R.K., Krystal,G., Teitelbaum,S.L. and Ross,F.P.
TITLE SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts
JOURNAL Nat. Med. 8 (9), 943-949 (2002)
PUBMED 12161749
REMARK GeneRIF: SHIP negatively regulates osteoclast formation and function and the absence of this enzyme results in severe osteoporosis
REFERENCE 7 (bases 1 to 4865)
AUTHORS Huber,M., Kalesnikoff,J., Reth,M. and Krystal,G.
TITLE The role of SHIP in mast cell degranulation and IgE-induced mast cell survival
JOURNAL Immunol. Lett. 82 (1-2), 17-21 (2002)
PUBMED 12008029
REMARK GeneRIF: a review of SHIP's role in mast cell degranulation and IgE-induced mast cell survival
REFERENCE 8 (bases 1 to 4865)
AUTHORS Kalesnikoff,J., Baur,N., Leitges,M., Hughes,M.R., Damen,J.E., Huber,M. and Krystal,G.
TITLE SHIP negatively regulates IgE + antigen-induced IL-6 production in mast cells by inhibiting NF-kappa B activity
JOURNAL J. Immunol. 168 (9), 4737-4746 (2002)
PUBMED 11971024
REMARK GeneRIF: SHIP negatively regulates IgE + antigen-induced IL-6 production in mast cells
REFERENCE 9 (bases 1 to 4865)
AUTHORS Wang,J.W., Howson,J.M., Ghansah,T., Desponts,C., Ninos,J.M., May,S.L., Nguyen,K.H., Toyama-Sorimachi,N. and Kerr,W.G.
TITLE Influence of SHIP on the NK repertoire and allogeneic bone marrow transplantation
JOURNAL Science 295 (5562), 2094-2097 (2002)
PUBMED 11896280
REMARK GeneRIF: influences the repertoire of NK receptors;plays an important role in two processes that limit the success of allogeneic marrow transplantation: graft rejection and graft-versus-host disease
REFERENCE 10 (bases 1 to 4865)
AUTHORS Tu,Z., Ninos,J.M., Ma,Z., Wang,J.W., Lemos,M.P., Desponts,C., Ghansah,T., Howson,J.M. and Kerr,W.G.
TITLE Embryonic and hematopoietic stem cells express a novel SH2-containing inositol 5'-phosphatase isoform that partners with the Grb2 adapter protein
JOURNAL Blood 98 (7), 2028-2038 (2001)
PUBMED 11567986
REFERENCE 11 (bases 1 to 4865)
AUTHORS Wolf,I., Lucas,D.M., Algate,P.A. and Rohrschneider,L.R.

TITLE Cloning of the genomic locus of mouse SH2 containing inositol 5-phosphatase (SHIP) and a novel 110-kDa splice isoform, SHIPdelta
JOURNAL Genomics 69 (1), 104-112 (2000)
PUBMED 11013080
REFERENCE 12 (bases 1 to 4865)
AUTHORS Lucas,D.M. and Rohrschneider,L.R.
TITLE A novel spliced form of SH2-containing inositol phosphatase is expressed during myeloid development
JOURNAL Blood 93 (6), 1922-1933 (1999)
PUBMED 10068665
REFERENCE 13 (bases 1 to 4865)
AUTHORS Liu,Q. and Dumont,D.J.
TITLE Molecular cloning and chromosomal localization in human and mouse of the SH2-containing inositol phosphatase, INPP5D (SHIP). Amgen EST Program
JOURNAL Genomics 39 (1), 109-112 (1997)
PUBMED 9027494
REFERENCE 14 (bases 1 to 4865)
AUTHORS Lioubin,M.N., Algate,P.A., Tsai,S., Carlberg,K., Aebersold,A. and Rohrschneider,L.R.
TITLE p150Ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity
JOURNAL Genes Dev. 10 (9), 1084-1095 (1996)
PUBMED 8654924
REFERENCE 15 (bases 1 to 4865)
AUTHORS Damen,J.E., Liu,L., Rosten,P., Humphries,R.K., Jefferson,A.B., Majerus,P.W. and Krystal,G.
TITLE The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 93 (4), 1689-1693 (1996)
PUBMED 8643691
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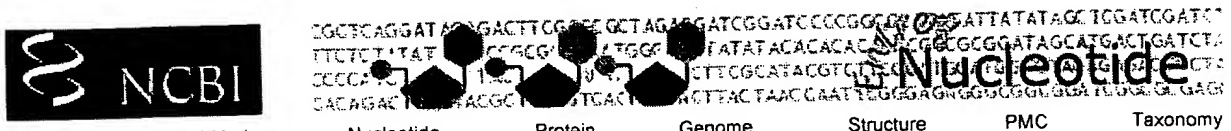

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REFERENCE 1 (bases 1 to 5273)
AUTHORS Krahn,A.K., Ma,K., Hou,S., Duronio,V. and Marshall,A.J.
TITLE Two distinct waves of membrane-proximal B cell antigen receptor signaling differentially regulated by Src homology 2-containing inositol polyphosphate 5-phosphatase
JOURNAL J. Immunol. 172 (1), 331-339 (2004)
PUBMED 14688341
REMARK GeneRIF: SHIP positively, rather than negatively, regulates in vitro membrane recruitment of pleckstrin homology domain-containing signaling proteins Bam32 and TAPP2, which therefore specify a distinct wave of phosphatidylinositol 3-kinase signaling in B cells.
REFERENCE 2 (bases 1 to 5273)
AUTHORS Jiang,X., Stuible,M., Chalandon,Y., Li,A., Chan,W.Y., Eisterer,W., Krystal,G., Eaves,A. and Eaves,C.
TITLE Evidence for a positive role of SHIP in the BCR-ABL-mediated transformation of primitive murine hematopoietic cells and in human chronic myeloid leukemia
JOURNAL Blood 102 (8), 2976-2984 (2003)
PUBMED 12829595
REMARK GeneRIF: SHIP expression appears to be differently altered in the early and late stages of differentiation of BCR-ABL-transformed cells
REFERENCE 3 (bases 1 to 5273)
AUTHORS Baran,C.P., Tridandapani,S., Helgason,C.D., Humphries,R.K., Krystal,G. and Marsh,C.B.
TITLE The inositol 5'-phosphatase SHIP-1 and the Src kinase Lyn negatively regulate macrophage colony-stimulating factor-induced Akt activity
JOURNAL J. Biol. Chem. 278 (40), 38628-38636 (2003)
PUBMED 12882960
REMARK GeneRIF: SHIP-1 and Lyn have roles in the negative regulation of M-CSF-R-induced Akt activation
REFERENCE 4 (bases 1 to 5273)
AUTHORS Galandrini,R., Tassi,I., Mattia,G., Lenti,L., Piccoli,M., Frati,L. and Santoni,A.
TITLE SH2-containing inositol phosphatase (SHIP-1) transiently translocates to raft domains and modulates CD16-mediated cytotoxicity in human NK cells

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PUBMED 8643691
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FEATURES Location/Qualifiers

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RNAi: Double-Stranded RNA Directs the ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals

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Summary

Double-stranded RNA (dsRNA) directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). Using a recently developed *Drosophila* in vitro system, we examined the molecular mechanism underlying RNAi. We find that RNAi is ATP dependent yet uncoupled from mRNA translation. During the RNAi reaction, both strands of the dsRNA are processed to RNA segments 21–23 nucleotides in length. Processing of the dsRNA to the small RNA fragments does not require the targeted mRNA. The mRNA is cleaved only within the region of identity with the dsRNA. Cleavage occurs at sites 21–23 nucleotides apart, the same interval observed for the dsRNA itself, suggesting that the 21–23 nucleotide fragments from the dsRNA are guiding mRNA cleavage.

Introduction

The term RNA interference, or "RNAi," was initially coined by Fire and coworkers (Fire et al., 1998) to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (for reviews see Fire, 1999; Hunter, 2000; Hunter, 1999; Montgomery and Fire, 1998; Sharp, 1999; Wagner and Sun, 1998). Their discovery built upon the previous, puzzling observation that sense and antisense RNA (asRNA) were equally effective in suppressing specific gene expression (Guo and Kemphues, 1995), a paradox resolved by the finding that small amounts of dsRNA contaminate sense and antisense preparations (Fire et al., 1998). RNAi has since been discovered in a wide variety of animals, including flies (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), trypanosomes (Ngo et al., 1998), planaria (Sánchez-Alvarado and Newmark, 1999), hydra (Lohmann et al., 1999), zebrafish (Wargelius et al., 1999), and mice (Wianny and Zernicka-Goetz, 2000), and appears to be related to gene silencing phenomena in plants ("cosuppression"; Vaucheret et al., 1998; Waterhouse et al., 1998, 1999; Baulcombe, 1999) and the fungus *Neurospora* ("quelling"; Cogoni et al., 1996; Cogoni and Macino, 1999a, 1999b).

RNAi occurs posttranscriptionally and involves mRNA degradation (Montgomery et al., 1998; Ngo et al., 1998). In addition to providing a powerful tool for creating gene-specific phenocopies of loss-of-function mutations, RNAi may also play an important biological role in protecting the genome against instability caused by the accumulation of transposons and repetitive sequences (Ketting et al., 1999; Tabara et al., 1999). In *C. elegans*, dsRNA blocks specific gene expression even when expressed by bacteria fed to the worms (Timmons and Fire, 1998). RNAi in animals may also represent an ancient antiviral response, just as posttranscriptional gene silencing appears to protect plants from viral infection (Baulcombe, 1999; Grant, 1999; Ratcliff et al., 1999). The breadth of RNAi-like processes suggests that RNAi may encompass gene silencing phenomena, including cellular strategies for gene regulation, well beyond the initial observation that dsRNA can produce RNAi.

Genetic screens in both *C. elegans* and *Neurospora* have identified genes required for RNAi (Cogoni and Macino, 1997; Tabara et al., 1999). Mutations in a subset of these genes, including *rde-2*, *rde-3*, *mut-2*, and *mut-7*, permit the mobilization of transposons in the worm germline (Ketting et al., 1999; Tabara et al., 1999; Grishok et al., 2000). A second class of mutants, including the *rde-1* and *rde-4* loci, are defective for RNAi but show no other phenotypic abnormalities (Tabara et al., 1999). The *rde-1* and *rde-4* genes are required for the initiation of heritable RNAi, a phenomenon in which RNAi established by injection of dsRNA in a worm leads to heritable gene silencing in the F2 generation and beyond (Grishok et al., 2000). In contrast, *rde-2* and *mut-7* are not required for the initiation of heritable interference but are required downstream in the tissue where the interference occurs. Mello and colleagues have proposed that *rde-1* and *rde-4* respond to dsRNA by producing a secondary extragenic agent that is used by the downstream genes *rde-2* and *mut-7* to target specific mRNAs for posttranscriptional gene silencing (Grishok et al., 2000). In this view, *rde-1* and *rde-4* act as initiators of RNAi, whereas *rde-2* and *mut-7* are effectors. These authors propose that other stimuli that lead to gene silencing, such as the accumulation of transposons or repetitive DNA in the genome or the introduction of a transgene, are interpreted by a separate set of initiator genes that produce the same secondary extragenic agent.

In *Neurospora*, the *qde-3* gene, which is required for quelling (a form of posttranscriptional silencing in which an endogenous gene is silenced by the introduction of a transgenic copy of the gene), may be an example of an initiator gene that responds to the presence of a transgene (Cogoni and Macino, 1999b). *qde-3* is a member of the RecQ DNA helicase family, which includes

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the human genes for Bloom's syndrome and Werner's syndrome.

One candidate for the secondary extragenic agent itself is the 25 nucleotide–long RNAs associated with posttranscriptional gene silencing in plants (Hamilton and Baulcombe, 1999). These RNAs, which correspond to both the sense and antisense strands of the silenced gene, are only detected in plants undergoing silencing. The level of expression of these short RNAs also correlates with the extent of gene silencing. It remains to be shown if the 25 nt RNAs are the actual agents or merely the products of gene silencing.

Two other genes implicated in posttranscriptional gene silencing, *qde-1* in *Neurospora* (Cogoni and Macino, 1999a) and *ego-1* in *C. elegans* (Smardon et al., 2000), are homologous to a tomato protein that displays RNA-directed RNA-polymerase activity in vitro (Schiebel et al., 1993a, 1993b, 1998). RNA-directed RNA polymerases have been implicated in the initial formation of the silencing agent or in the amplification of dsRNA. Amplification of injected dsRNA by an endogenous RNA-directed RNA polymerase would help explain how a very small number of dsRNA molecules can inactivate a much larger population of mRNAs and how the dsRNA can apparently persist in the animal for many days and even into subsequent generations. *ego-1* mutants are defective for RNAi for maternally, but not zygotically, expressed mRNAs. Interestingly, *ego-1* is also required for germline development in *C. elegans* (Qiao et al., 1995).

Biochemical analysis of RNAi has become possible with the development of an in vitro *Drosophila* embryo lysate that recapitulates dsRNA-dependent silencing of gene expression (Tuschl et al., 1999). In the in vitro system, dsRNA—but not sense or asRNA—targets a corresponding mRNA for degradation yet does not affect the stability of an unrelated control mRNA. Furthermore, preincubation of the dsRNA in the lysate potentiates its activity for target mRNA degradation, suggesting that the dsRNA must be converted to an active form by binding proteins in the extract or by covalent modification (Tuschl et al., 1999).

Here, we use the in vitro system to analyze the requirements of RNAi and to determine the fate of the dsRNA and the mRNA. RNAi in vitro requires ATP but does not require either mRNA translation or recognition of the 7-methyl-guanosine cap of the targeted mRNA. The dsRNA but not single-stranded RNA is processed in vitro to a population of 21–23 nt species. Deamination of adenosines within the dsRNA does not appear to be required for formation of the 21–23 nt RNAs. Furthermore, we find that the mRNA is cleaved only in the region corresponding to the sequence of the dsRNA and that the mRNA is cleaved at 21–23 nt intervals, strongly suggesting that the 21–23 nt fragments from the dsRNA are targeting the cleavage of the mRNA.

Results and Discussion

RNAi Requires ATP

Drosophila embryo lysates faithfully recapitulate RNAi (Tuschl et al., 1999). Previously, dsRNA-mediated gene silencing was monitored by measuring the synthesis of

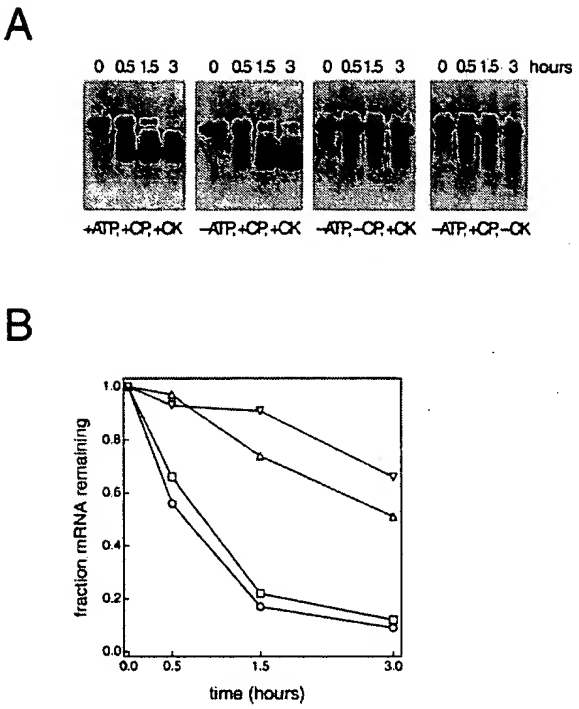

Figure 1. RNAi Requires ATP

(A) Denaturing agarose-gel analysis of 5′-^{32}P-radiolabeled *Rr*-luc mRNA incubated for the times indicated in an in vitro RNAi reaction with or without ATP, creatine phosphate (CP), or creatine kinase (CK), as indicated below each panel.

(B) Quantitation of the data in (A). Circles, +ATP, +CP, +CK; squares, -ATP, +CP, +CK; triangles, -ATP, -CP, +CK; inverted triangles, -ATP, +CP, -CK.

luciferase protein from the targeted mRNA. Thus, these RNAi reactions contained an ATP-regenerating system, needed for the efficient translation of the mRNA. To test if ATP was, in fact, required for RNAi, the lysates were depleted for ATP by treatment with hexokinase and glucose, which converts ATP to ADP, and RNAi was monitored directly by following the fate of ^{32}P-radiolabeled *Renilla reniformis* luciferase (*Rr*-luc) mRNA (Figure 1). Treatment with hexokinase and glucose reduced the endogenous ATP level in the lysate from 250 μM to below 10 μM (data not shown). ATP regeneration required both exogenous creatine phosphate and creatine kinase, which acts to transfer a high-energy phosphate from creatine phosphate to ADP. When ATP-depleted extracts were supplemented with either creatine phosphate or creatine kinase separately, no RNAi was observed. Therefore, RNAi requires ATP in vitro. When ATP, creatine phosphate, and creatine kinase were all added together to reactions containing the ATP-depleted lysate, dsRNA-dependent degradation of the *Rr*-luc mRNA was restored (Figure 1). The addition of exogenous ATP was not required for efficient RNAi in the depleted lysate, provided that both creatine phosphate and creatine kinase were present, demonstrating that the endogenous concentration (250 μM) of adenosine nucleotide is sufficient to support RNAi. RNAi with a *Photinus pyralis* luciferase (*Pp*-luc) mRNA was also ATP dependent (data not shown).

The stability of the *Rr*-luc mRNA in the absence of

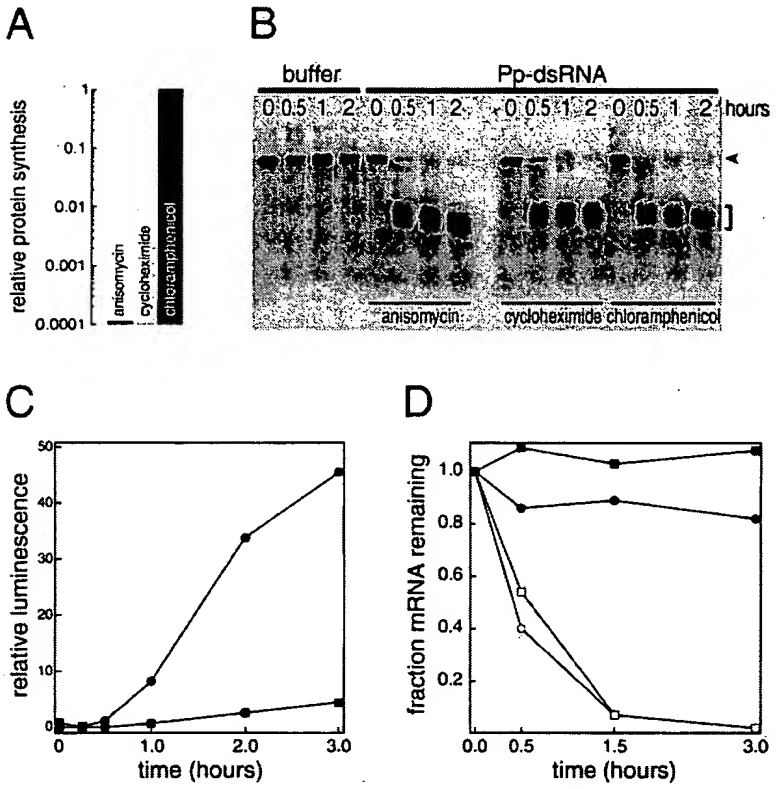

Figure 2. RNAi Does Not Require mRNA Translation

(A) Protein synthesis, as reflected by luciferase activity produced after incubation of *Rr*-luc mRNA in the in vitro RNAi reaction for 1 hr, in the presence of the protein synthesis inhibitors anisomycin, cycloheximide, or chloramphenicol, relative to a reaction without any inhibitor.

(B) Denaturing agarose-gel analysis of 5′-^{32}P-radiolabeled *Pp*-luc mRNA after incubation for the indicated times in a standard RNAi reaction with and without protein synthesis inhibitors. The arrowhead indicates the position of full-length mRNA in the gel, and the bracket marks the position of stable, 5′ cleavage products.

(C) Translation of 7-methyl-guanosine- and adenosine-capped *Pp*-luc mRNAs (circles and squares, respectively) in the RNAi reaction in the absence of dsRNA, as measured by luciferase activity produced in a 1 hr incubation.

(D) Incubation in an RNAi reaction of uniformly ^{32}P-radiolabeled 7-methyl-guanosine-capped *Pp*-luc mRNA (circles) and adenosine-capped *Pp*-luc mRNA (squares), in the presence (open symbols) and absence (filled symbols) of 505 bp *Pp*-luc dsRNA.

Rr-dsRNA was reduced in ATP-depleted lysates relative to that observed when the energy regenerating system was included, but decay of the mRNA under these conditions did not display the rapid decay kinetics characteristic of RNAi in vitro, nor did it generate the stable mRNA cleavage products characteristic of dsRNA-directed RNAi (data not shown). These experiments do not establish if the ATP requirement for RNAi is direct, implicating ATP in one or more steps in the RNAi mechanism, or indirect, reflecting a role for ATP in maintaining high concentrations of another nucleoside triphosphate in the lysate.

Translation Is Not Required for RNAi In Vitro

The requirement for ATP suggested that RNAi might be coupled to mRNA translation, a highly energy-dependent process. To test this possibility, various inhibitors of protein synthesis were added to the reaction. We tested the eukaryotic translation inhibitors anisomycin, an inhibitor of initial peptide bond formation, cycloheximide, an inhibitor of peptide chain elongation, and puromycin, a tRNA mimic that causes premature termination of translation (Cundliffe, 1981). Each of these inhibitors reduced protein synthesis in the *Drosophila* lysate by more than 1,900-fold (Figure 2A; data not shown). In contrast, chloramphenicol, an inhibitor of *Drosophila* mitochondrial protein synthesis (Page and Orr-Weaver, 1997), had no effect on translation in the lysates (Figure 2A). Despite the presence of anisomycin, cycloheximide, or chloramphenicol, RNAi proceeded at normal efficiency (Figure 2B). Puromycin also did not perturb efficient RNAi (data not shown). Thus, protein synthesis is not required for RNAi in vitro.

Translational initiation is an ATP-dependent process that involves recognition of the 7-methyl guanosine cap of the mRNA (Merrick and Hershey, 1996; Kozak, 1999). The *Drosophila* lysate used to support RNAi in vitro also recapitulates the cap dependence of translation: *Pp*-luc mRNA with a 7-methyl-guanosine cap was translated greater than 10-fold more efficiently than was the same mRNA with an A(5′)ppp(5′)G cap (Figure 2C). Both RNAs were equally stable in the *Drosophila* lysate, showing that this difference in efficiency cannot be merely explained by more rapid decay of the mRNA with an adenosine cap (also see Gebauer et al., 1999). Although the translational machinery can discriminate between *Pp*-luc mRNAs with 7-methyl-guanosine and adenosine caps, the two mRNAs were equally susceptible to RNAi in the presence of *Pp*-dsRNA (Figure 2D). These results suggest that steps in cap recognition are not involved in RNAi.

dsRNA Is Processed to 21–23 Nucleotide Species

RNAs 25 nt in length are generated from both the sense and antisense strands of genes undergoing posttranscriptional gene silencing in plants (Hamilton and Baulcombe, 1999). We find that dsRNA is also processed to small RNA fragments (Figures 3A and 3B). When incubated in lysate, approximately 15% of the input radioactivity of both the 501 bp *Rr*-dsRNA and the 505 bp *Pp*-dsRNA appeared in 21 to 23 nt RNA fragments. Because the dsRNAs are more than 500 bp in length, the 15% yield of fragments implies that multiple 21–23 nt RNAs are produced from each full-length dsRNA molecule. No other stable products were detected. The small RNA species were produced from dsRNAs in which both

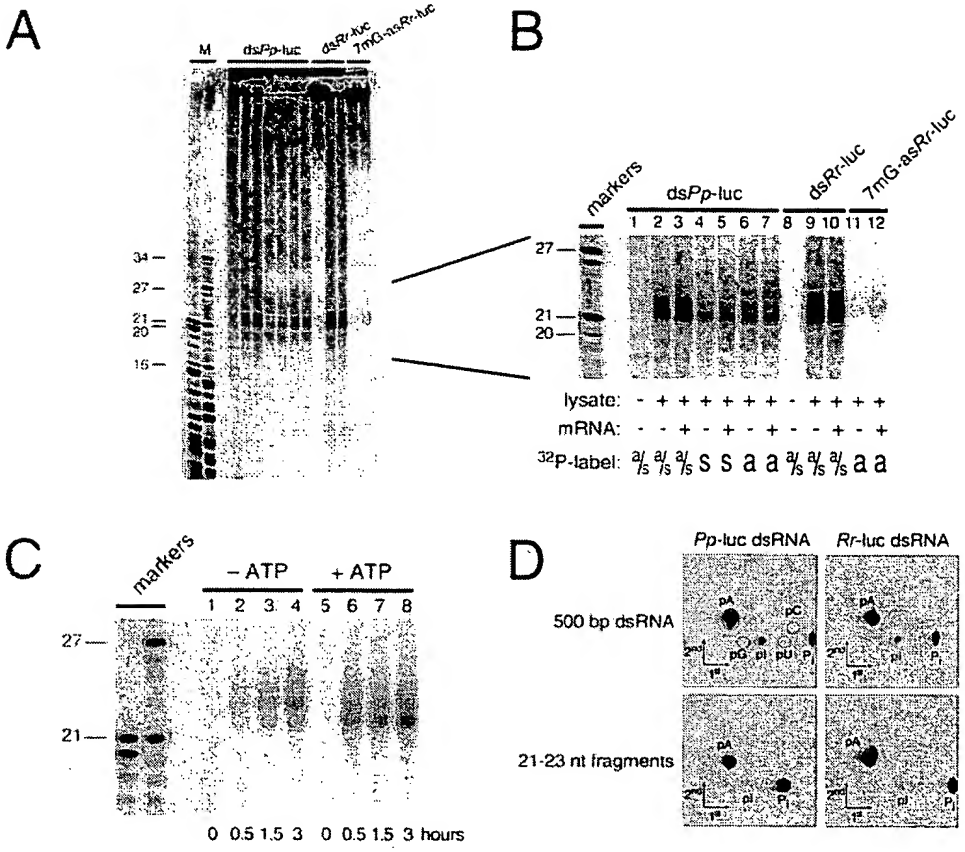

Figure 3. 21–23 nt RNA Fragments Are Produced upon Incubation of dsRNA in *Drosophila* Embryo Lysate

(A) Denaturing acrylamide-gel analysis of the products formed in a 2 hr incubation of uniformly ^{32}P-radiolabeled dsRNAs or capped asRNA in lysate under standard RNAi conditions, in the presence or absence of target mRNAs.

(B) An enlargement of the portion of the gel in (A) corresponding to 17 to 27 nt. For *Pp*-dsRNA, the sense (lanes 4 and 5) or the antisense (lanes 6 and 7) or both strands (lanes 1, 2, and 3) were labeled. For *Rr*-luc dsRNA, both strands were radioactive (lanes 8, 9, and 10).

(C) An enlargement of the 17 to 27 nt region of a gel showing the products formed upon incubation of uniformly ^{32}P-radiolabeled dsRNAs in lysate without and with ATP.

(D) Adenosine deamination in full-length dsRNA and the 21–23 nt RNA species assessed by two-dimensional thin-layer chromatography. Circles correspond to positions of unlabeled 5′-nucleotide monophosphate standards visualized under UV light. Inorganic phosphate (P_i) was produced by the degradation of mononucleotides by phosphatases that contaminate commercially available nuclease P1 (Auxilien et al., 1996).

strands were uniformly ^{32}P-radiolabeled (Figure 3B, lanes 2, 3, 9, and 10). Formation of the 21–23 nt RNAs from the dsRNA did not require the presence of the corresponding mRNA (Figure 3B, compare lane 2 with lane 3 and lane 9 with lane 10), demonstrating that the small RNA species is generated by processing of the dsRNA, rather than as a product of dsRNA-targeted mRNA degradation. We note that 22 nucleotides corresponds to two turns of an A-form RNA–RNA helix.

When dsRNAs radiolabeled within either the sense or the antisense strand were incubated with lysate in a standard RNAi reaction, 21–23 nt RNAs were generated with comparable efficiency (Figure 3B, compare lanes 4 and 6). These data support the idea that the 21–23 nt RNAs are generated by symmetric processing of the dsRNA. A variety of data support the idea that the 21–23 nt RNA is efficiently generated only from dsRNA and is not the consequence of an interaction between single-stranded RNA and the dsRNA. First, a ^{32}P-radiolabeled 505 nt *Pp*-luc sense RNA or asRNA was not efficiently converted to the 21–23 nt product when it was incubated with 5 nM nonradioactive 505 bp *Pp*-dsRNA (data not shown). Second, in the absence of mRNA, a 501 nt 7-methyl-guanosine-capped *Rr*-asRNA produced only a barely detectable amount of 21–23 nt RNA (Figure 3B, lane 11; capped single-stranded RNAs are as stable in the lysate as dsRNA [Tuschl et al., 1999]), probably due to a small amount of dsRNA contaminating the antisense preparation. However, when *Rr*-luc mRNA was included in the reaction with the ^{32}P-radiolabeled, capped *Rr*-asRNA, a small amount of 21–23 nt product was generated, corresponding to 4% of the amount of 21–23 nt RNA produced from an equimolar amount of *Rr*-dsRNA. This result is unlikely to reflect the presence of contaminating dsRNA in the *Rr*-asRNA preparation, since significantly more product was generated from the asRNA in the presence of the *Rr*-luc mRNA than in the absence (compare lanes 12 and 11). Instead, the data suggest that asRNA can interact with the complementary mRNA sequences to form dsRNA in the reaction and that the

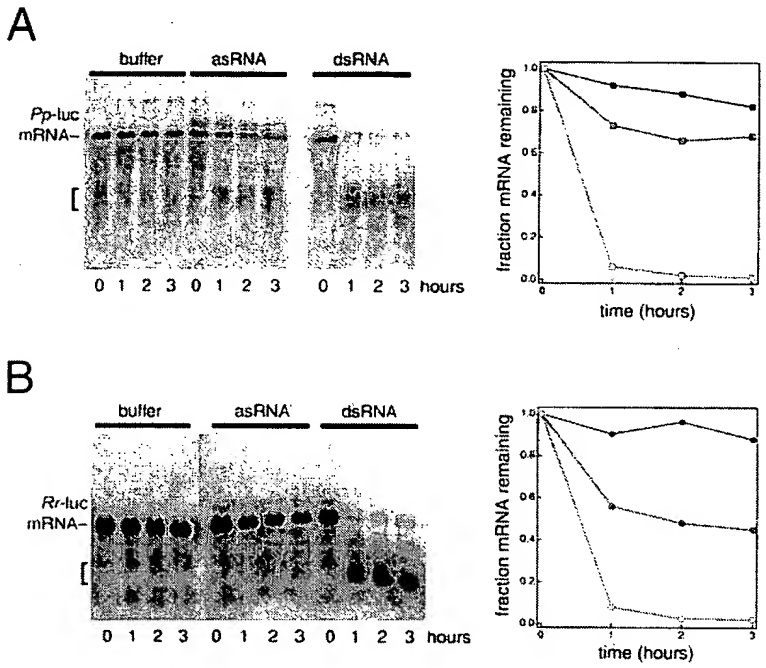

Figure 4. asRNA Causes a Small Amount of RNAi In Vitro

(A) Denaturing agarose-gel analysis of *Pp*-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt *Pp*-asRNA, or 505 bp *Pp*-dsRNA for the times indicated.

(B) The same analysis for the *Rr*-luc mRNA. Quantitation of the gel data in both (A) and (B) is given to the right of each panel. Buffer, black symbols; asRNA, blue symbols; dsRNA, red symbols.

resulting dsRNA is subsequently processed to the small RNA species. *Rr*-asRNA can support a low level of bona fide RNAi in vitro (see below), consistent with this explanation.

We next asked if production of the 21–23 nt RNAs from dsRNA required ATP (Figure 3C). When the 505 bp *Pp*-dsRNA was incubated in a lysate depleted for ATP by treatment with hexokinase and glucose, 21–23 nt RNA was produced (lanes 1–4, "–ATP"), albeit six times slower than when ATP was regenerated in the depleted lysate by the inclusion of creatine kinase and creatine phosphate (lanes 5–8, "+ ATP"). Therefore, ATP may not be required for production of the 21–23 nt RNA species but may instead simply enhance its formation. Alternatively, ATP may be required for processing of the dsRNA, but at a concentration less than that remaining after hexokinase treatment. We do not yet understand the molecular basis for the slower mobility of the small RNA fragments generated in the ATP-depleted lysate.

Wagner and Sun (1998) and Sharp (1999) have speculated that the requirement for dsRNA in gene silencing by RNAi reflects the involvement of a dsRNA-specific adenosine deaminase in the process. dsRNA adenosine deaminases unwind dsRNA by converting adenosine to inosine, which does not base pair with uracil. dsRNA adenosine deaminases function in the posttranscriptional editing of mRNA (reviewed by Bass, 1997). To test for the involvement of dsRNA adenosine deaminase in RNAi, we examined the degree of conversion of adenosine to inosine in the 501 bp *Rr*-luc and 505 bp *Pp*-luc dsRNAs after incubation with *Drosophila* embryo lysate in a standard in vitro RNAi reaction (Figure 3D). We also determined the degree of adenosine deamination in the 21–23 nt species. The full-length dsRNA radiolabeled with [^{32}P]-adenosine was incubated in the lysate, and both the full-length dsRNA and the 21–23 nt RNA products were purified from a denaturing acrylamide gel, cleaved to mononucleotides with nuclease P1, and analyzed by two-dimensional thin-layer chromatography.

A significant fraction of the adenosines in the full-length dsRNA were converted to inosine after 2 hr (3.1% and 5.6% conversion for *Pp*-luc and *Rr*-luc dsRNAs, respectively). In contrast, only 0.4% (*Pp*-dsRNA) or 0.7% (*Rr*-dsRNA) of the adenosines in the 21–23 nt species were deaminated. These data imply that fewer than 1 in 27 molecules of the 21–23 nt RNA species contain an inosine. Therefore, it is unlikely that dsRNA-dependent adenosine deamination within the 21–23 nt species is required for its production.

asRNA Generates a Small Amount of RNAi In Vitro

When mRNA was ^{32}P-radiolabeled within the 5′-7-methyl-guanosine cap, stable 5′ decay products accumulated during the RNAi reaction (see, for example, Figures 1A and 2B). Such stable 5′ decay products were observed for both the *Pp*-luc and *Rr*-luc mRNAs when they were incubated with their cognate dsRNAs (indicated by the brackets in Figures 4A and 4B). Previously, we reported that efficient RNAi does not occur when asRNA is used in place of dsRNA (Tuschl et al., 1999). Nevertheless, mRNA was measurably less stable when incubated with asRNA than with buffer (Figures 4A and 4B). This was particularly evident for the *Rr*-luc mRNA: approximately 90% of the RNA remained intact after a 3 hr incubation in lysate, but only 50% when asRNA was added. Less than 5% remained when dsRNA was added. Interestingly, the decrease in mRNA stability caused by asRNA was accompanied by the formation of a small amount of the stable 5′ decay products characteristic of the RNAi reaction with dsRNA. This finding parallels the observation that a small amount of 21–23 nt product formed from the asRNA when it was incubated with the mRNA (see above) and lends strength to the idea that asRNA can enter the RNAi pathway, albeit inefficiently.

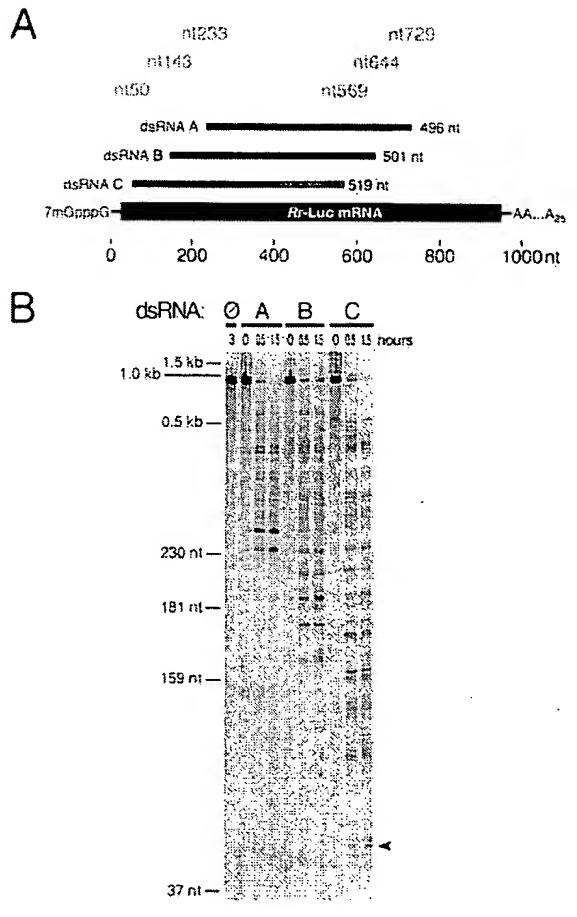

Figure 5. The dsRNA Determines the Boundaries of the Cleavage Products

(A) Schematic of the positions of the three dsRNAs, A, B, and C, relative to the *Rr*-luc mRNA.

(B) Denaturing acrylamide-gel analysis of the stable, 5′ cleavage products produced after incubation of the *Rr*-luc mRNA for the indicated times with each of the three dsRNAs; A, B, and C, or with buffer (zero with strikethrough). The positions of RNA markers radiolabeled within their 5′ cap is shown at left. The arrowhead denotes a faint cleavage site that is indicated with an open blue circle in Figure 6B.

mRNA Cleavage Sites Are Determined by the Sequence of the dsRNA

The sites of mRNA cleavage were examined using three different dsRNAs, "A," "B," and "C," displaced along the *Rr*-luc sequence by approximately 100 nt. The positions of these relative to the *Rr*-luc mRNA sequence are shown (Figure 5A). Each of the three dsRNAs was incubated in a standard RNAi reaction with *Rr*-luc mRNA ^{32}P-radiolabeled within the 5′ cap (Figure 5B). In the absence of dsRNA, no stable 5′ cleavage products were detected for the mRNA, even after 3 hr of incubation in lysate. In contrast, after a 20 min incubation, each of the three dsRNAs produced a ladder of bands corresponding to a set of mRNA cleavage products characteristic for that particular dsRNA. For each dsRNA, the stable, 5′ mRNA cleavage products were restricted to the region of the *Rr*-luc mRNA that corresponded to the dsRNA (Figures 5B and 6). For dsRNA A, the lengths of the 5′ cleavage products ranged from 236 to just under ~750 nt; dsRNA A spans nucleotides 233 to 729 of the *Rr*-luc mRNA. Incubation of the mRNA with dsRNA B produced mRNA 5′ cleavage products ranging in length from 150 to ~600 nt; dsRNA B spans nucleotides 143 to 644 of the mRNA. Finally, dsRNA C produced mRNA cleavage products from 66 to ~500 nt in length. This dsRNA spans nucleotides 50 to 569 of the *Rr*-luc mRNA. Therefore, the dsRNA not only provides specificity for the RNAi reaction, selecting which mRNA from the total cellular mRNA pool will be degraded, but also determines the boundaries of cleavage along the mRNA sequence.

The mRNA Is Cleaved at 21–23 Nucleotide Intervals

To gain further insight into the mechanism of RNAi, we mapped the positions of several mRNA cleavage sites for each of the three dsRNAs (Figure 6). Remarkably, most of the cleavages occurred at 21–23 nt intervals (Figure 6A). This spacing is especially striking in light of our observation that the dsRNA is processed to a 21–23 nt RNA species and the finding of Hamilton and Baulcombe that a 25 nt RNA correlates with posttranscriptional gene silencing in plants (Hamilton and Baulcombe, 1999). Of the 16 cleavage sites we mapped (two for dsRNA A, five for dsRNA B, and nine for dsRNA C), all but two reflect the 21–23 nt interval. One of the two exceptional cleavages was a weak cleavage site produced by dsRNA C (indicated by an arrowhead in Figure 5B and an open blue circle in Figure 6B). This cleavage occurred 32 nt 5′ to the next cleavage site. The other exception is particularly intriguing. After four cleavages spaced 21–23 nt apart, dsRNA C caused cleavage of the mRNA just 9 nt 3′ to the previous cleavage site (Figures 6A and 6B, red arrowhead). This cleavage occurred in a run of seven uracil residues and appears to "reset" the ruler for cleavage; the next cleavage site was 21–23 nt 3′ to the exceptional site. The three subsequent cleavage sites that we mapped were also spaced 21–23 nt apart. Curiously, of the sixteen cleavage sites mapped for the three different dsRNAs, fourteen occur at uracil residues. We do not yet understand the significance of this finding, but it suggests that mRNA cleavage is determined by a process that measures 21–23 nt intervals and that has a sequence preference for cleavage at uracil. In preliminary experiments, the 21–23 nt RNA species produced by incubation of ~500 bp dsRNA in the lysate caused sequence-specific interference in vitro when isolated from an acrylamide gel and added to a new RNAi reaction in place of the full-length dsRNA (our unpublished data).

A Model for dsRNA-Directed mRNA Cleavage

Our biochemical data, together with recent genetic experiments in *C. elegans* and *Neurospora* (Cogoni and Macino, 1999a; Ketting et al., 1999; Tabara et al., 1999; Grishok et al., 2000), suggest a model for how dsRNA targets mRNA for destruction (Figure 7). In this model, the dsRNA is first cleaved to 21 to 23 nt long fragments in a process likely to involve genes such as the *C. elegans* loci *rde-1* and *rde-4*. The resulting fragments, probably as short asRNAs bound by RNAi-specific proteins, would then pair with the mRNA and recruit a nuclease that cleaves the mRNA. Alternatively, strand exchange could occur in a protein–RNA complex that transiently

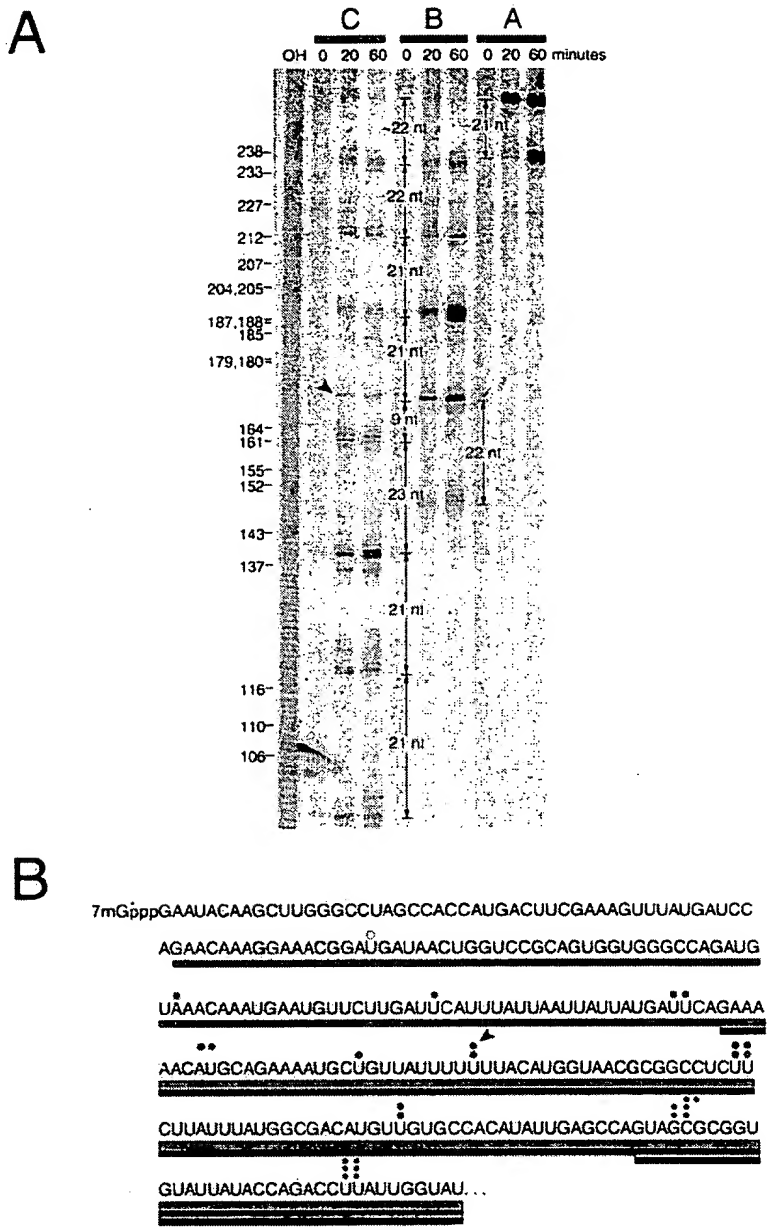

Figure 6. The mRNA Is cleaved in 21–23 nt Intervals

(A) High-resolution denaturing acrylamide-gel analysis of a subset of the 5′ cleavage products described in Figure 5B. The positions of some of the partial T1 digestion products of *Rr*-luc mRNA are indicated at left. "OH" marks the lane in which a partial base-hydrolysis ladder was loaded.

(B) The cleavage sites in (A) mapped onto the first 267 nt of the *Rr*-luc mRNA. The blue bar below the sequence indicates the position of dsRNA C, and blue circles indicate the position of cleavage sites caused by this dsRNA. The green bar denotes the position of dsRNA B, and green circles, the cleavage sites. The magenta bar indicates the position of dsRNA A, and magenta circles, the cleavages. An exceptional cleavage within a run of seven uracils is marked with a red arrowhead in both (A) and (B).

holds a 21–23 nt dsRNA fragment close to the mRNA. Separation of the two strands of the dsRNA following fragmentation might be assisted by an ATP-dependent RNA helicase, explaining the ATP enhancement of 21–23 nt RNA production we observed.

We envision that each small RNA fragment produces one, or at most two, cleavages in the mRNA, perhaps at the 5′ or 3′ ends of the 21–23 nt fragment. The small RNAs may be amplified by an RNA-directed RNA polymerase such as that encoded by the *ego-1* gene in *C. elegans* (Smardon et al., 2000) or the *qde-1* gene in *Neurospora* (Cogoni and Macino, 1999a), producing long-lasting posttranscriptional gene silencing in the absence of the dsRNA that initiated the RNAi effect. Heritable RNAi in *C. elegans* requires the *rde-1* and *rde-4* genes to initiate but not to persist in subsequent generations. The *rde-2*, *rde-3*, and *mut-7* genes in *C. elegans* are required in the tissue where RNAi occurs but are not required for initiation of heritable RNAi (Grishok et al., 2000). These "effector" genes (Grishok et al., 2000) are likely to encode proteins functioning in the actual selection of mRNA targets and in their subsequent cleavage. ATP may be required at any of a number of steps during RNAi, including complex formation on the dsRNA, strand dissociation during or after dsRNA cleavage, pairing of the 21–23 nt RNAs with the target mRNA, mRNA cleavage, and recycling of the targeting complex. Testing these ideas with the in vitro RNAi system will be an important challenge for the future.

Experimental Procedures

In Vitro RNAi

In vitro RNAi reactions and lysate preparation were as described previously (Tuschl et al., 1999) except that the reaction contained 0.03 μg/ml creatine kinase, 25 mM creatine phosphate (Fluka), and 1 mM ATP. Creatine phosphate was freshly dissolved at 500 mM in

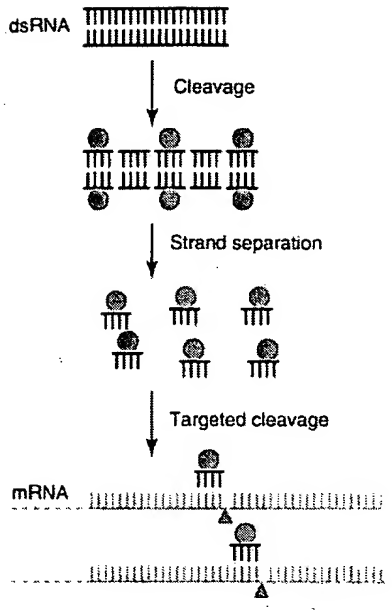

Figure 7. Proposed Model for RNAi

RNAi is envisioned to begin with cleavage of the dsRNA to 21–23 nt products by a dsRNA-specific nuclease, perhaps in a multiprotein complex. These short dsRNAs might then be dissociated by an ATP-dependent helicase, possibly a component of the initial complex, to 21–23 nt asRNAs that could then target the mRNA for cleavage. The short asRNAs are imagined to remain associated with the RNAi-specific proteins (ochre circles) that were originally bound by the full-length dsRNA, thus explaining the inefficiency of asRNA to trigger RNAi in vivo and in vitro. Finally, a nuclease (triangles) would cleave the mRNA.

water for each experiment. GTP was omitted from the reactions, except in Figures 2 and 3.

RNA Synthesis

Pp-luc and *Rr*-luc mRNAs and *Pp*- and *Rr*-dsRNAs (including dsRNA B in Figure 6) were synthesized by in vitro transcription as described previously (Tuschl et al., 1999). To generate transcription templates for dsRNA C, the 5′ sense RNA primer was gcgtaatacgactcactata GAACAAAGGAAACGGATGAT and the 3′ sense RNA primer was GAAGAAGTTATTCTCCAAAA; the 5′ asRNA primer was gcgtaatac gactcactataGAAGAAGTTATTCTCCAAAA and the 3′ asRNA primer was GAACAAAGGAAACGGATGAT. For dsRNA A, the 5′ sense RNA primer was gcgtaatacgactcactataGTAGCGCGGTGTATTATACC and the 3′ sense RNA primer was GTACAACGTCAGGTTTACCA; the 5′ asRNA primer was gcgtaatacgactcactataGTACAACGTCAGGTTT ACCA and the 3′ asRNA primer was GTAGCGCGGTGTATTATACC (lowercase, T7 promoter sequence).

mRNAs were 5′ end labeled using guanylyl transferase (Gibco/BRL), S-adenosyl methionine (Sigma), and α-^{32}P-GTP (3000 Ci/mmol; New England Nuclear) according to the manufacturer's directions. Radiolabeled RNAs were purified by poly(A) selection using the Poly(A) Tract III kit (Promega). Nonradioactive 7-methyl-guanosine- and adenosine-capped RNAs were synthesized in in vitro transcription reactions with a 5-fold excess of 7-methyl-G(5′)ppp(5′)G or A(5′)ppp(5′)G relative to GTP. Cap analogs were purchased from New England Biolabs.

ATP Depletion and Protein Synthesis Inhibition

ATP was depleted by incubating the lysate for 10 min at 25°C with 2 mM glucose and 0.1 U/μl hexokinase (Sigma). Protein synthesis inhibitors were purchased from Sigma and dissolved in absolute ethanol as 250-fold concentrated stocks. The final concentrations of inhibitors in the reaction were anisomycin, 53 μg/ml; cycloheximide, 100 μg/ml; and chloramphenicol, 100 mg/ml. Relative protein synthesis was determined by measuring the activity of *Rr* luciferase protein produced by translation of the *Rr*-luc mRNA in the RNAi reaction after 1 hr as described previously (Tuschl et al., 1999).

Analysis of dsRNA Processing

Internally α-^{32}P-ATP-labeled dsRNAs (505 bp *Pp*-luc or 501 *Rr*-luc) or 7-methyl-guanosine-capped *Rr*-luc antisense RNA (501 nt) were incubated at 5 nM final concentration in the presence or absence of unlabeled mRNAs in *Drosophila* lysate for 2 hr in standard conditions. Reactions were stopped by the addition of 2× proteinase K buffer and deproteinized as described previously (Tuschl et al., 1999). Products were analyzed by electrophoresis in 15% or 18% polyacrylamide sequencing gels. Length standards were generated by complete RNase T1 digestion of α-^{32}P-ATP-labeled 501 nt *Rr*-luc sense RNA and asRNA.

For analysis of mRNA cleavage, 5′-^{32}P-radiolabeled mRNA (described above) was incubated with dsRNA as described previously (Tuschl et al., 1999) and analyzed by electrophoresis in 5% (Figure 5B) and 6% (Figure 6C) polyacrylamide sequencing gels. Length standards included commercially available RNA size standards (FMC Bioproducts) radiolabeled with guanylyl transferase as described above and partial base hydrolysis and RNase T1 ladders generated from the 5′-radiolabeled mRNA.

Deamination Assay

Internally α-^{32}P-ATP-labeled dsRNAs (5 nM) were incubated in *Drosophila* lysate for 2 hr at standard conditions. After deproteinization, samples were run on 12% sequencing gels to separate full-length dsRNAs from the 21–23 nt products. RNAs were eluted from the gel slices in 0.3 M NaCl overnight, ethanol precipitated, collected by centrifugation, and redissolved in 20 μl water. The RNA was hydrolyzed into nucleoside 5′ phosphates with nuclease P1 (10 μl reaction containing 8 μl RNA in water, 30 mM KOAc [pH 5.3], 10 mM $ZnSO_4$, and 10 μg or 3 units nuclease P1, for 3 hr at 50°C). Samples (1 μl) were cospotted with nonradioactive 5′ mononucleotides (0.05 O. D. units [A_{260}] of pA, pC, pG, pI, and pU) on cellulose HPTLC plates (EM Merck) and separated in the first dimension in isobutyric acid/25% ammonia/water (66/1/33, v/v/v) and in the second dimension in 0.1 M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol (100/60/2, v/w/v; Silberklang et al., 1979). Migration of the nonradioactive internal standards was determined by UV shadowing.

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Note Added in Proof

Recently, Hammond et al. have shown that ~25 nt RNAs are generated in cultured *Drosophila* S2 cells transfected with *cyclin E* dsRNA (Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. [2000]. Nature *404*, 293–296.

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Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference

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SUMMARY

Specific mRNA degradation mediated by double-stranded RNA (dsRNA), which is termed RNA interference (RNAi), is a useful tool with which to study gene function in several systems. We report here that in mouse oocytes, RNAi provides a suitable and robust approach to study the function of dormant maternal mRNAs. *Mos* (originally known as c-*mos*) and tissue plasminogen activator (tPA, *Plat*) mRNAs are dormant maternal mRNAs that are recruited during oocyte maturation; translation of *Mos* mRNA results in the activation of MAP kinase. dsRNA directed towards *Mos* or *Plat* mRNAs in mouse oocytes effectively results in the specific reduction of the targeted mRNA in both a time- and concentration-dependent manner. Moreover, dsRNA is more potent than either sense or antisense RNAs. Targeting the *Mos* mRNA results in inhibiting the appearance of MAP kinase activity and can result in parthenogenetic activation. *Mos* dsRNA, therefore, faithfully phenocopies the *Mos* null mutant. Targeting the *Plat* mRNA with *Plat* dsRNA results in inhibiting production of tPA activity. Finally, effective reduction of the *Mos* and *Plat* mRNA is observed with stoichiometric amounts of *Mos* and *Plat* dsRNA, respectively.

Key words: Maternal mRNA, RNA interference, mouse oocyte, *Mos*, *Plat*

INTRODUCTION

'Omne vivum ex ovo' (All living things come from eggs), which is attributed to William Harvey, is probably the first articulation of the current view that the program for early development is established during oogenesis. During oogenesis in the mouse, oocytes grow and acquire the ability to resume and complete meiosis (acquisition of meiotic competence) (Sorensen and Wassarman, 1976; Wickramasinghe et al., 1991), as well as the ability to be fertilized and develop to term (acquisition of developmental competence) (Eppig and O'Brien, 1996). Meiotic maturation and egg activation are accompanied by the recruitment of many maternal mRNAs (Schultz et al., 1979; Schultz and Wassarman, 1977; Van Blerkom, 1981), and presumably some of these direct the synthesis of proteins that are required for the formation of a fertilizable egg that is capable of developing to term. One such mRNA is the *Mos* mRNA. The mobilization of the *Mos* mRNA results in the ultimate activation of mitogen-activated protein (MAP) kinase, whose activity is required to maintain arrest at metaphase II (Gebauer and Richter, 1997; Sagata, 1997); oocytes lacking the *Mos* gene mature to metaphase II but then undergo spontaneous activation, i.e., they emit the second polar body and form a pronucleus (Colledge et al., 1994; Hasimoto et al., 1994). The tissue plasminogen activator (tPA, *Plat*) mRNA is another maternal mRNA that is recruited during oocyte maturation (Huarte et al., 1987; Vassalli et al., 1989). Although tPA is synthesized during maturation and secreted following fertilization, and then becomes associated with a cell-surface receptor on the embryo (Carroll et al., 1993), *Plat* knockout mice are viable and fertile, but do display mild perturbations in phenotype, e.g., retardation in neuronal migration (Seeds et al., 1999).

To date, an antisense RNA approach has been the most widely used method to assess the function of maternal mRNAs that are recruited during oocyte maturation. Nevertheless, this approach has problems. For example, an antisense RNA approach has been used to assess the role of *Mos* mRNA recruitment during maturation. The phenotypes observed range from permitting germinal vesicle breakdown but inhibiting emission of the first polar body (Paules et al., 1989; Zhao et al., 1991), to emission of the first polar body but entering interphase instead of proceeding to and arresting at metaphase II (O'Keefe et al., 1989). In contrast, the phenotype of a *Mos* null mutant generated by homologous recombination is that the oocytes proceed to metaphase II, but meiotic arrest is not maintained and the eggs spontaneously undergo parthenogenetic activation (Colledge et al., 1994; Hasimoto et al., 1994). This discrepancy between the phenotypes observed by the antisense approach with that of a 'true' knockout potentially confounds the use of antisense RNA to study the function of a dormant maternal mRNA. Antisense RNA can

also target and destroy the *Plat* mRNA. The efficacy of destruction of the untranslated *Plat* mRNA, however, appears restricted to antisense RNA directed towards the 3′ UTR (Strickland et al., 1988). Antisense RNAs directed at other portions of the *Plat* mRNA are far less effective and can form hybrids only following maturation and the concomitant recruitment of the *Plat* mRNA. Thus, the efficacy of this approach is compromised by the appropriate selection of the region of the mRNA to be targeted, and this can only be determined experimentally and not a priori.

Recently, RNA interference (RNAi), which employs double-stranded RNA (dsRNA), has been shown to ablate potently the targeted mRNA in a variety of species (Sanchez-Alvardado and Newmark, 1999; Fire et al., 1998; Kennerdell and Carthew, 1998; Li et al., 2000; Lohmann et al., 1999; Misquitta and Paterson, 1999; Ngo et al., 1998; Wargelius et al., 1999). The destruction of the targeted mRNA by dsRNA occurs prior to translation (Fire, 1999; Montgomery et al., 1998; Sharp, 1999; Zamore et al., 2000), and targets exon sequences; dsRNA directed against intron sequences is ineffective (Fire et al., 1998). Genetic approaches in *Caenorhabditis elegans* have identified genes with homology to eIF-2C, RNase D, and RNA-directed RNA polymerase (Ketting et al., 1999; Tabara et al., 1999; Smardon et al., 2000) that are involved in the RNAi-mediated pathway of mRNA degradation. Very recent studies suggest that a nuclease involved in the destruction of the targeted mRNA contains an essential RNA component containing approx. 25-nucleotide RNAs that are homologous to the dsRNA (Hammond et al., 2000). The processing of the dsRNA to these fragments does not require the presence of the targeted mRNA, and the targeted mRNA is cleaved only in the regions of identity to the dsRNA and at sites that are 21-23 nucleotides apart (Zamore et al., 2000).

We report here that dsRNA directed towards *Mos* and *Plat* mRNAs in mouse oocytes effectively results in the specific reduction of the targeted mRNA in both a time- and concentration-dependent manner. Moreover, dsRNA is more potent than either sense or antisense RNA. Targeting the *Mos* mRNA results in inhibiting the appearance of MAP kinase activity, as well as promoting parthenogenetic activation of the treated cells, and targeting *Plat* mRNA results in inhibiting production of tPA activity. Effective reduction of the *Mos* and *Plat* mRNA is observed with stoichiometric amounts of *Mos* and *Plat* dsRNA, respectively. While these studies were in progress, a paper appeared that has reported that oocytes injected with *Mos* dsRNA undergo egg activation, as evidenced by pronucleus formation (Wianny and Zernicka-Goetz, 2000).

MATERIALS AND METHODS

dsRNA preparation

For *Mos* amplification, a pair of primers was designed based on the cDNA sequence (Accession number J00372). The sequence of upstream *Mos* primer was 5′-CCATCAAGCAAGTAAACAAG-3′ and the downstream *Mos* primer was 5′-AGGGTGATTCCAAAAGA-GTA-3′. These primers generated a PCR product that was 535 bp in length and corresponded to the 3′ end of the coding region and the beginning of the 3′UTR. Likewise, for *Plat* amplification a pair of primers was designed based on the cDNA sequence (Accession number J03520). The sequence of the upstream *Plat* primer was 5′-CATGGGCAAGCGTTACACAG-3′ and the downstream *Plat* primer was 5′-CAGAGAAGAATGGAGACGAT-3′. These primers generated a PCR product that was 650 bp in length and corresponded to the middle part of the coding region.

To generate template for transcription in vitro, 5 μg of liver total RNA were reverse transcribed with Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's instructions using oligo-dT as the primer. PCR amplification conditions for both *Mos* and *Plat* were as follows: initial denaturation at 94°C for 4 minutes was followed by 36 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute and the final cycle had an extended incubation at 72°C for 7 minutes followed by decrease to 4°C. All PCR reactions were performed in either a PE2400 or PE9600 PCR thermocycler.

Gel-purified primary PCR products were diluted 1:500 and re-amplified to produce specific templates to generate sense and antisense transcripts by transcription in vitro. To do this, primers were made that contained an SP6 promoter attached to the 5′ end of both the forward and reverse primers. Following PCR under the above conditions, the secondary PCR products were purified using a Nucleospin Extraction Kit (Clontech). The template (500-1000 ng) was then transcribed with SP6 RNA polymerase (Promega) in order to obtain the corresponding sense and antisense RNAs.

The in vitro transcription products were resolved following electrophoresis in 1.5% NuSieve LM agarose (FMC, Rockland, ME, USA) and the bands corresponding to the sense and antisense single-stranded RNA were purified according to the manufacturer's protocol. Equimolar amounts of sense and antisense RNA were then annealed in 1 mM Tris-HCl (pH 7.5), containing 1 mM EDTA, or in DEPC-treated water supplemented with 5% RNasin (Promega); similar results were obtained using either procedure. Typically, 2-4 μg of RNA in 30 μl were mixed and heated in 500 ml of boiling water for 1 minute. The sample, still in the water bath, was allowed to cool to room temperature over the course of several hours. The dsRNA was phenol extracted, ethanol precipitated, washed in 75% ethanol and then dissolved in water. Samples were stored in water at −80°C prior to use.

RNA isolation and RT-PCR

RNA was isolated from oocytes and prepared for RT-PCR as previously described (Temeles et al., 1994). In each case, 0.125 pg of rabbit β-globin mRNA/oocyte was added prior to RNA isolation. The globin mRNA serves as an internal standard for the efficiency of the RT-PCR reactions (Temeles et al., 1994). For each set of gene-specific primers the linear region of semi-log plots of the amount of PCR product as a function of cycle number was determined and a cycle number for each primer pair was selected that was in this linear range; the amount of PCR product under these conditions is proportional to the number of cells used (Manejwala et al., 1991). This method permits the comparison of relative changes in the abundance of a particular transcript (Ho et al., 1995; Latham et al., 1994; Temeles et al., 1994).

Following reverse transcription two oocyte equivalents were used as a template for each PCR reaction. PCR products were labeled with [α-^{32}P]dCTP (Amersham, 0.25 μCi per 50 μl reaction). PCR amplification conditions for both *Mos* and *Plat* were as follows: initial denaturation at 94°C for 2 minutes was followed by 28 (*Plat*) or 31 (*Mos*) cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, followed by 4°C until the samples were removed. PCR amplification conditions for globin: initial denaturation at 94°C for 2 minutes was followed by 24 cycles of 94°C for 10 seconds and 62°C for 15 seconds followed by final 4°C. After PCR, the products were subjected to electrophoresis in an 8% polyacrylamide gel. The gel was dried under vacuum for 1 hour at 80°C, exposed in phosphorimager cassette for 4 to 24 hours and the signal was quantified using the Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Oocyte collection, microinjection and culture

Fully grown, germinal vesicle (GV)-intact oocytes were obtained from pregnant mare's serum gonadotropin (PMSG)-primed six-week-old female CF-1 mice (Harlan) and freed of attached cumulus cells, as previously described (Schultz et al., 1983). The collection medium was bicarbonate-free minimal essential medium (Earle's salt) supplemented with polyvinylpyrrolidone (3 mg/ml) and 25 mM Hepes, pH 7.3. Germinal vesicle breakdown was inhibited by including 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX). The oocytes were transferred into CZB medium (Chatot et al., 1989) containing 0.2 mM IBMX (CZB+IBMX) and cultured in an atmosphere of 5% CO_2 in air at 37°C. Oocytes were microinjected in bicarbonate-free CZB containing 10 mM Hepes and 0.2 mM IBMX with 5 pl of the corresponding solution; the injections were performed as previously described (Kurasawa et al., 1989). The concentration of the undiluted stock solution was 0.2 μg/μl and injection of 5 pl of either *Mos* or *Plat* dsRNA corresponds to 1.7×10^6 and 1.4×10^6 molecules, respectively. When single-stranded RNA was injected, it was diluted to a concentration such that injection of 5 pl corresponded to the same number of molecules as when dsRNA was injected. In experiments in which either enzyme activity or phenotype was assayed, microinjected oocytes were cultured in CZB+IBMX for 10 or 20 hours. They were then washed through ten drops of IBMX-free CZB and cultured in CZB until oocyte collection and lysis. In experiments in which mRNA levels were measured, the oocytes were kept in medium containing IBMX for 10, 20, or 40 hours until they were collected and processed for RNA isolation.

tPA assay

tPA activity was assayed by zymography of single oocytes. Immobilon-P (Millipore) was soaked in methanol for 1 minute and then rinsed four times with 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl. The wet membrane was placed on Whatman paper soaked with this buffer and both were transferred to a 96-well dot blot apparatus (Milliblot Systems, Millipore) with the Immobilon-P facing upwards. A 96-well template was then placed on the stage and single oocytes were transferred in 1-2 μl of CZB medium to the middle of where the wells would form. The apparatus was completely assembled and the wet membrane with the oocytes was then exposed to vacuum suction for 2 minutes. The apparatus was then disassembled and the membrane was immediately applied on the detection gel; the detection gel, which contained 40 μg/ml of plasminogen (Fluka), was prepared as previously described (Vassalli et al., 1984). Zymograms were developed for 12-64 hours at 37°C, scanned with a black background and the lysed area was estimated using the ImageQuant software (Molecular Dynamics).

Histone H1 and MBP kinase assay

The activities of both histone H1 and myelin basic protein (MBP) kinases were determined in single eggs as follows: single eggs were transferred in 1.5 μl of culture medium into a tube containing 3.5 μl of double kinase lysis buffer (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM *p*-nitrophenyl phosphate, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 5 mM EGTA). The tubes were immediately frozen on dry ice and stored at −80°C until the assay was performed. The kinase reaction was initiated by the addition of 5 μl of double kinase buffer (24 mM *p*-nitrophenyl phosphate, 90 mM β-glycerophosphate, 24 mM $MgCl_2$, 24 mM EGTA, 0.2 mM EDTA, 4.6 mM sodium orthovanadate, 4 mM NaF, 1.6 mM dithiothreitol, 60 μg/ml aprotinin, 60 μg/ml leupeptin, 2 mg/ml polyvinyl alcohol, 2.2 μM protein kinase A inhibitor peptide (Sigma), 40 mM 3-(n-morpholino) propanesulfonic acid (MOPS), pH 7.2, 0.6 mM ATP, 2 mg/ml histone (type III-S, Sigma), 0.5 mg/ml MBP) with 500 μCi/ml [γ-^{32}P]ATP (3000 Ci/mmol; Amersham). To determine the background level of phosphorylation for H1 and MBP, 5 μl of double kinase lysis buffer was added instead of the egg lysate. The reaction was conducted for 30 minutes at 30°C and terminated by the addition of 10 μl double-strength concentrated SDS-PAGE sample buffer (Laemmli, 1970) and boiling for 3 minutes. Following SDS-PAGE, the 15% gel was fixed in 10% acetic acid/30% methanol, dried and exposed to a phosphorimager screen for 16 to 24 hours. Scanning and quantification of the signal were performed using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). For each experiment, the mean value of H1 or MBP kinase activities for oocytes microinjected with sense *Mos* RNA was arbitrarily set as 100% and the values obtained in the other groups of eggs were expressed relative to this amount.

RESULTS

Preparation of dsRNA

dsRNA for either *Mos* or *Plat* was prepared by hybridizing equimolar amounts of gel-purified, single-stranded sense and antisense transcripts that were generated by transcription in vitro of appropriate templates containing an SP6 promoter. In each case, the RNA was directed towards a coding portion of the transcript. Following hybridization, the single-stranded RNAs were essentially totally converted to dsRNA, as evidenced by the absence of any visible staining in the region of the gel that corresponded to single-stranded species (Fig. 1). The quantitative nature of hybridization permitted use of the dsRNA without any need for gel purification of the dsRNA species.

Effect of *Mos* and *Plat* dsRNA on *Mos* and *Plat* mRNA levels in mouse oocytes

Mos and *Plat* are two maternal mRNAs that are recruited during oocyte maturation (see Introduction). We selected to target the *Mos* mRNA since a *Mos* null oocyte has a defined phenotype, i.e., the oocyte matures to metaphase II, but rather than arresting at metaphase II, it undergoes spontaneous egg activation. In addition, it is possible to measure MAP kinase activity in a single oocyte; MAP kinase activity reflects the mobilization of *Mos* mRNA (see below). We also selected to target *Plat* mRNA, which like *Mos*, is a moderately abundant mRNA; it has been estimated that an oocyte contains approx. 10,000 transcripts each of *Mos* and *Plat* (Huarte et al., 1987;

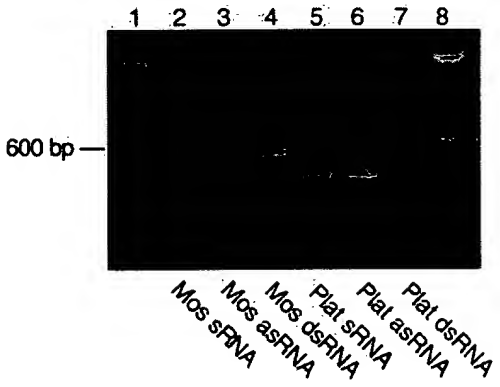

Fig. 1. Generation of *Mos* or *Plat* dsRNA. Sense or antisense *Mos* or *Plat* RNAs were produced by transcription in vitro and then gel purified. Equimolar concentrations of sense and antisense RNA were then hybridized and a portion of the reaction analyzed by electrophoresis. Shown is an ethidium bromide-stained gel demonstrating the quantitative formation of dsRNA. Lanes 1 and 8, 100 bp ladder.

Keshet et al., 1988). In addition, it is also possible to measure tPA activity in single oocytes.

Oocytes were injected with approx. 10^6 molecules of either *Plat* or *Mos* dsRNA that was directed towards the coding region of each transcript; this corresponds to approx. 10 nM final concentration (see Discussion). The oocytes were then cultured in medium containing IBMX to inhibit resumption of meiosis; a decrease in cAMP is associated with resumption of meiosis and including the membrane-permeable phosphodiesterase inhibitor IBMX in the medium prevents the decrease in cAMP and thus the resumption of meiosis (Schultz et al., 1983). Following culture, RNA was isolated and the relative amount of *Plat* and *Mos* transcripts were determined by a semi-quantitative RT-PCR assay that permits quantification of relative changes in transcript abundance. Prior to RNA isolation, a known amount of rabbit globin mRNA was added; this served as a control for RNA recovery, and for the efficiency of the RT-PCR (Temeles et al., 1994).

Oocytes injected with *Mos* dsRNA displayed a marked reduction in the amount of *Mos* transcript (approx. 80%), relative to water-injected or uninjected controls (Fig. 2, compare lane 2 with lanes 4 and 5). Likewise, oocytes injected with *Plat* dsRNA displayed an approx. 90% reduction in the amount of *Plat* transcript relative to the control (Fig. 2, lane 3). Specificity of this effect was demonstrated by the finding that *Mos* dsRNA did not reduce the abundance of *Plat* mRNA, and reciprocally, that *Plat* dsRNA did not reduce the abundance of *Mos* mRNA (Fig. 2). Results of these experiments indicated that the machinery for RNAi-mediated degradation of the targeted endogenous mRNA is present and functions in mouse oocytes.

Effect of *Mos* and *Plat* sense and antisense RNA on *Mos* and *Plat* mRNA levels in mouse oocytes

In other systems, antisense RNA can be ineffective. For example, injection of *C. elegans* with antisense RNA directed towards the *unc*-22 gene does not result in the mutant twitching phenotype, whereas dsRNA does (Fire et al., 1998). Nevertheless, antisense RNA can be effective in degrading oocyte mRNAs (Strickland et al., 1988). Accordingly, we determined the effect of sense and antisense *Mos* and *Plat* RNA on targeting the cognate oocyte transcript.

Oocytes were injected with approx. 10^6 copies of either sense, antisense or dsRNA, and incubated for 20 hours in medium containing IBMX, before the RNA was isolated and transcript abundance determined. As anticipated, dsRNA directed towards either *Mos* or *Plat* mRNA resulted in the reduction of the targeted mRNA, whereas the untargeted transcript remained essentially intact (Fig. 3A). As also anticipated, injection of sense RNA resulted in little, if any, decrease in the abundance of either the targeted or nontargeted mRNA. Injection of either *Mos* or *Plat* antisense RNA, however, did result in a decrease in the targeted, but not in the nontargeted, mRNA (Fig. 3A). Little, if any decrease in the targeted mRNA was observed when the amount of injected *Mos* or *Plat* antisense RNA was decreased by 10-fold (Fig. 3B). In contrast, this amount of dsRNA was effective in decreasing the amount of the targeted mRNA (Fig. 3B), e.g., the *Mos* dsRNA resulted in an approx. 85% decrease in *Mos* mRNA, and *Plat* dsRNA resulted in an approx. 30% decrease in *Plat* mRNA. Results of these experiments suggest that dsRNA is more effective in targeting mRNAs than antisense RNA.

Concentration- and time-dependence of dsRNA directed towards *Mos* and *Plat* mRNAs

In the experiments described above, the oocytes were injected

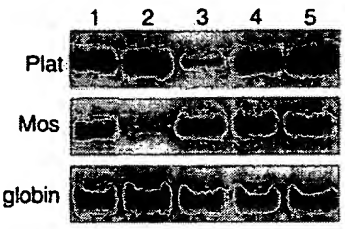

Fig. 2. Effect of *Mos* and *Plat* dsRNA on the relative abundance of *Mos* and *Plat* transcripts. Oocytes were injected with approx. 10^6 molecules of either *Mos* or *Plat* dsRNA and then cultured in the presence of IBMX for 20 hours. RNA was isolated and the relative amount of *Mos* and *Plat* transcripts were determined by RT-PCR, as described in Materials and Methods. The intensity of the globin band permits comparison of the different lanes, as it normalizes for RNA recovery, and for the efficiency of the RT-PCR part of the assay. Lane 1, relative amount of transcripts present in uninjected oocytes at t=0 hours; lane 2, relative amount of transcripts at t=20 hours in oocytes injected with *Mos* dsRNA; lane 3, relative amount of transcripts at t=20 hours in oocytes injected with *Plat* dsRNA; lane 4, relative amount of transcripts at t=20 hours in water-injected oocytes; lane 5, relative amount of transcripts at t=20 hours in uninjected oocytes.

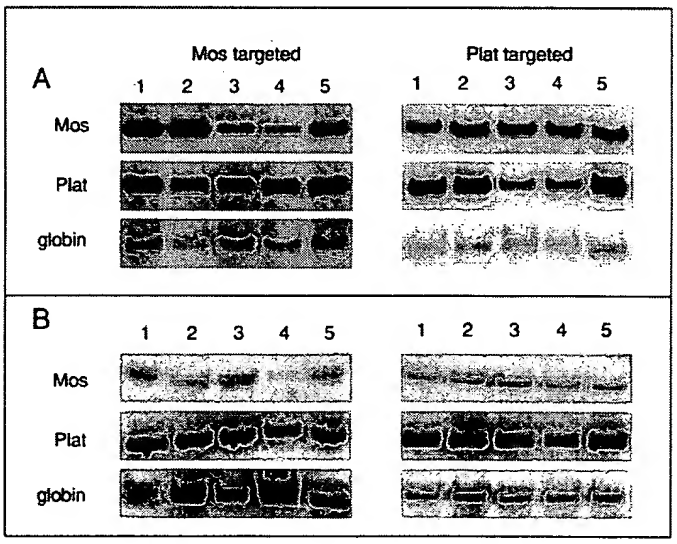

Fig. 3. Effect of *Mos* and *Plat* sense, antisense, and dsRNA on the relative abundance of *Mos* and *Plat* transcripts. (A) Oocytes were injected with approx. 10^6 molecules of either *Mos* or *Plat* sense, antisense or dsRNA and then cultured in the presence of IBMX for 20 hours. RNA was then isolated and the relative amount of *Mos* and *Plat* transcripts were determined by RT-PCR as described in the Materials and Methods. (B) Oocytes were injected with 10^5 molecules of either *Mos* or *Plat* sense, antisense or dsRNA and then processed as described in A. Lane 1, relative amount of transcripts present in water-injected oocytes; lane 2, relative amount of transcripts in sense RNA-injected oocytes; lane 3, relative amount of transcripts in antisense RNA-injected oocytes; lane 4, relative amount of transcripts in dsRNA-injected oocytes; lane 5, relative amount of transcripts in uninjected oocytes.

with approx. 10^6 molecules of dsRNA and cultured for 20 hours prior to determining the relative amount of targeted transcript. In order to determine further characteristics of the RNAi effect, the concentration- and time-dependence of this effect were determined. Oocytes were injected with 10^6, 10^4, or 10^2 molecules of either *Mos* or *Plat* dsRNA, and then incubated for 10, 20, or 40 hours prior to determining the relative abundance of the endogenous *Mos* and *Plat* transcripts (Fig. 4). For both *Mos* and *Plat* dsRNA-injected oocytes, the targeted message was destroyed in both a time- and concentration-dependent manner. In all cases, the nontargeted mRNA was not destroyed (data not shown).

Injection of 10^6 or 10^4 molecules of *Mos* dsRNA resulted in a substantial reduction in the amount of *Mos* mRNA, such that by 20 hours more than 75% of the mRNA was degraded; 10^2 molecules of injected *Mos* dsRNA had little, if any effect over the 40-hour timecourse. Although 10^6 molecules of injected *Plat* dsRNA also dramatically reduced the amount of *Plat* mRNA, the kinetics of *Plat* mRNA degradation were slower, when compared with those obtained for *Mos* dsRNA. In addition, 10^4 molecules of *Plat* dsRNA was not nearly effective as 10^4 molecules of *Mos* dsRNA. Similar to *Mos* dsRNA, the 100 molecules of injected *Plat* dsRNA was ineffective in reducing the amount of *Plat* mRNA.

Effect of Mos dsRNA on MAP kinase and p34^{cdc2}/cyclin B kinase activities

The experiments described above documented that both *Mos* and *Plat* dsRNA could result in the degradation of the targeted mRNA in a concentration- and time-dependent manner. We next demonstrated that the reduction of the targeted mRNA resulted in loss of formation of the encoded protein. The translation of *Mos* mRNA that initiates at the onset of oocyte maturation results in synthesis of MOS, which in turn activates MAP kinase kinase by phosphorylation (Gebauer and Richter, 1997; Sagata, 1997). MAP kinase kinase, which is a dual-specificity kinase, then phosphorylates MAP kinase on Thr183 and Tyr185 in the mammal, which in turn results in MAP kinase activation (Nishida and Gotoh, 1993). MAP kinase, which is a component of cytostatic factor (CSF) and is required to maintain metaphase II arrest, is frequently assayed by measuring the phoshorylation of MBP. Concomitant with germinal vesicle breakdown is the activation of p34^{cdc2}/cyclin B kinase (MPF) (Gebauer and Richter, 1997; Sagata, 1997), which is routinely assayed by phosphorylation of histone H1. In the mouse, MPF activation precedes MAP kinase activation by about 1-2 hours, and both activities reach maximal levels in the metaphase II-arrested egg (Verlhac et al., 1993). Following fertilization, MPF activity declines prior to MAP kinase activity (Moos et al., 1995; Verlhac et al., 1993).

Oocytes were injected with either *Mos* dsRNA, antisense RNA or sense RNA and cultured for 20 hours in IBMX-containing medium, then transferred to IBMX-free medium. The oocytes then matured to metaphase II, at which time both MAP and p34^{cdc2}/cyclin B kinase activities were assayed simultaneously in single eggs. As expected, sense RNA did not inhibit either kinase activity when compared with uninjected or water-injected eggs (data not shown). In contrast, both *Mos* antisense and dsRNA inhibited MAP kinase activity, although a greater degree of inhibition was observed with dsRNA (Fig. 5). This result was consistent with *Mos* dsRNA eliciting a greater decrease in *Mos* mRNA than *Mos* antisense RNA (Fig. 3). Although *Mos* antisense RNA did inhibit MAP kinase activity, the level of p34^{cdc2}/cyclin B kinase, i.e., histone H1 kinase, was reduced by only about 25% relative to control sense-injected or uninjected oocytes, while a 70% decrease was observed in the dsRNA-injected oocytes. This reduced amount of histone H1 kinase activity in the dsRNA-injected oocytes

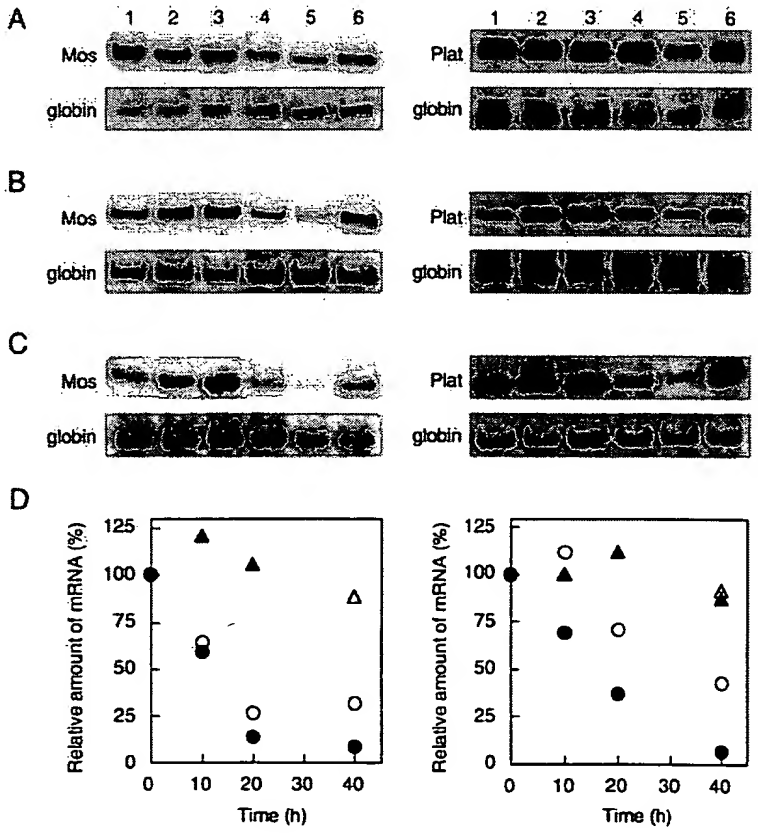

Fig. 4. Concentration- and time-dependence of *Mos* and *Plat* dsRNA-mediated reduction of the targeted mRNA. Oocytes were injected with 10^2 (lane 3), 10^4 (lane 4) or 10^6 (lane 5) molecules of either *Mos* or *Plat* dsRNA, and the relative abundance of either the *Mos* or *Plat* transcript was assayed after either 10 hours (A), 20 hours (B) or 40 hours (C) of culture in medium containing IBMX. Lane 1, relative amount of transcripts present in uninjected oocytes at t=0; lane 2, relative amount of transcripts in water-injected oocytes; lane 6, relative amount of transcripts in uninjected oocytes. (D) Quantification of the relative amount of *Mos* or *Plat* transcripts. The data are normalized to the amount present in the uninjected oocytes at the appropriate time following culture in IBMX-containing medium and all data are normalized to the globin signal, i.e., the ratio of the pixel volume of the transcript to that of the globin is set as 100%. (●), 10^6 molecules injected; (○), 10^4 molecules injected; (▲), 10^2 molecules injected; (△), the amount of *Plat* transcript present in oocytes injected with 10^6 molecules of *Mos* dsRNA or the amount of *Mos* transcript in oocytes injected with 10^6 molecules of *Plat* dsRNA. In order to keep the y-axis of similar scale, the value for oocytes injected with 10^2 molecules of *Mos* dsRNA and analyzed at 40 hours is not shown.

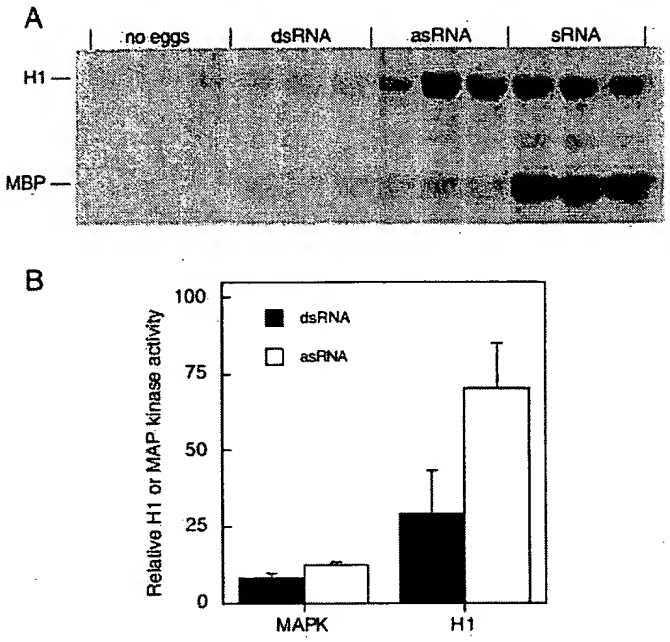

Fig. 5. Effect of *Mos* sense, antisense, and dsRNA on MAP kinase and MPF activities. Oocytes were injected with 10^6 molecules of either *Mos* sense (s), antisense (as) or dsRNA (ds), and then incubated for 20 hours in IBMX-containing medium. The oocytes were then transferred to IBMX-free medium and allowed to mature to metaphase II (about 18 hours), at which time single oocytes were assayed for both MAP kinase activity and MPF activity using MBP and histone H1, respectively as substrates. (A) Region of autoradiogram showing where phosphorylated histone H1 and MBP migrate. (B) Relative amount of kinase activity. The data have been normalized to that present in oocytes injected with sense RNA and this value does not differ from uninjected oocytes (data not shown). The data are expressed as the mean±s.e.m. and represent a total of 15, 15 and 13 dsRNA-, asRNA- and sRNA-injected oocytes, respectively.

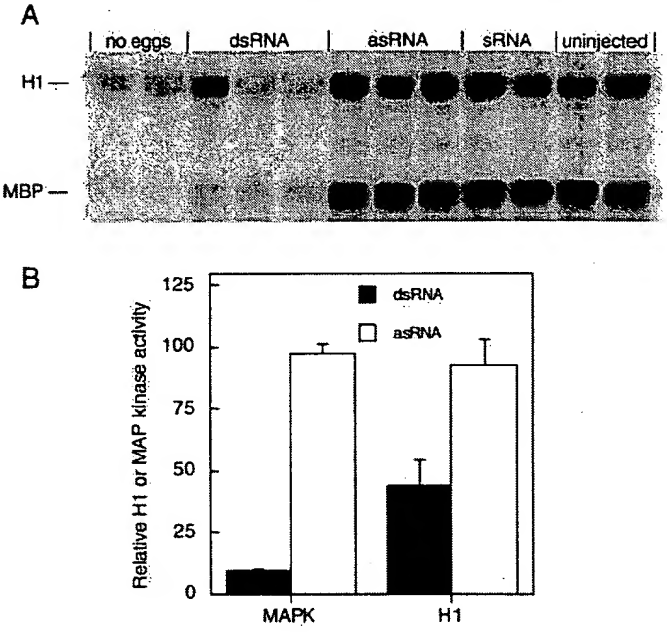

Fig. 6. Effect of *Mos* sense, antisense and dsRNA on MAP kinase and MPF activities. Oocytes were injected with 10^5 molecules of either *Mos* sense (s), antisense (as) or dsRNA (ds) and the experiment was then conducted as described in the legend to Fig. 5. (A) Region of autoradiogram showing where phosphorylated histone H1 and MBP migrate. (B) Relative amount of kinase activity. The data have been normalized to that present in oocytes injected with sense RNA and this value does not differ from uninjected oocytes. The data are expressed as the mean±s.e.m. and represent a total of 10, 10 and 6 dsRNA-, asRNA- and sRNA-injected oocytes.

was a consequence that in numerous cases these eggs underwent spontaneous egg activation, which results in a decrease in histone H1 kinase activity. In contrast, the antisense-injected oocytes never underwent egg activation.

Although *Mos* antisense RNA, which did result in a decrease in *Mos* mRNA, could inhibit MAP kinase activation, the results presented in Fig. 3 indicate that dsRNA is a more potent inhibitor than antisense RNA. Accordingly, oocytes were injected with 1/10 the amount of *Mos* sense, antisense, or dsRNA, cultured for 20 hours in IBMX-containing medium and then matured to metaphase II by transferring them to IBMX-free medium. The eggs were then assayed for both MAP and $p34^{cdc2}$/cyclin B kinase activities simultaneously in individual eggs. Whereas both *Mos* sense and antisense RNA did not inhibit the appearance of MAP kinase activity ($p34^{cdc2}$/cyclin B kinase activity was also high in these eggs), *Mos* dsRNA still elicited a dramatic inhibition in MAP kinase activity, and a corresponding decease in $p34^{cdc2}$/cyclin B kinase activity (Fig. 6). These results strengthen the conclusion that *Mos* dsRNA is more potent than *Mos* antisense RNA in promoting the reduction of the endogenous *Mos* mRNA.

Effect of Plat dsRNA on tPA activity

tPA is synthesized during oocyte maturation and its activity can be assayed in single oocytes by zymography (Huarte et al., 1985, 1987; Strickland et al., 1988). We observed only a single band (M_r 72,000) when metaphase II-arrested eggs were used; no activity was observed in GV-stage oocytes (data not shown). The presence of a single activity responsible for generating the lytic zone permitted analysis of tPA activity by simply spotting an oocyte/egg on a membrane, which was then overlaid with an agarose gel containing non-fat dry milk and plasminogen. The area of the lytic zone was linear as a function of time after a lag, which probably reflected the time to activate the zymogen cascade and degrade enough substrate to be visible to the eye (Fig. 7).

We assayed the effect of *Plat* sense, antisense and dsRNA on tPA activity in matured oocytes. Oocytes were injected with approx. 10^6 molecules of each RNA and cultured in IBMX-containing medium for 20 hours prior to initiating maturation by transfer to IBMX-free medium. Culture for 18 hours resulted in the production of metaphase II-arrested eggs that were then assayed for tPA activity. Injection of either antisense RNA or dsRNA resulted in a dramatic reduction in the amount of tPA activity, when compared with sense-injected oocytes (Fig. 8, black bars). The ability of *Plat* antisense RNA to inhibit the production of tPA activity following maturation is consistent with its ability to target the destruction of *Plat* mRNA (see Fig. 3A and Strickland et al., 1988), as well as its ability to inhibit translation of the *Plat* mRNA (Strickland et al., 1988). Nevertheless, injection of *Plat* sense RNA also modestly inhibited the production of tPA activity, although to

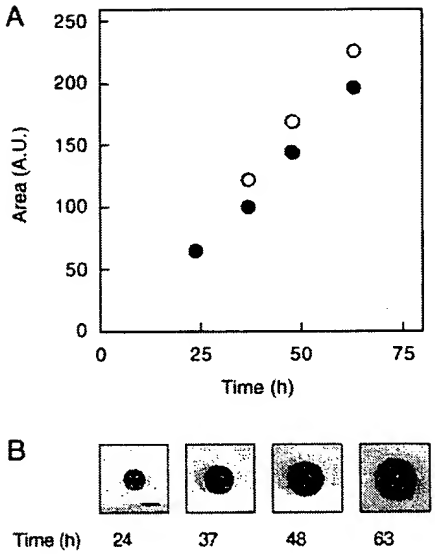

Fig. 7. Zymographic assay of tPA activity in single eggs. (A) Time-dependent increase in the area of the lytic zone of two eggs. (B) Photomicrographs of the lytic zone as a function of time of a single egg. Scale bar: 3 mm.

a lesser extent than either *Plat* antisense or dsRNA (Fig. 8, black bars). We observed that *Mos* sense RNA, which doesn't target the *Plat* mRNA, also resulted in a 50% decrease in tPA activity but had no inhibitory effect on the activation of MAP kinase (data not shown). The molecular basis underlying this inhibitory effect of sense RNA on the generation of tPA activity remains unresolved.

The results presented in Fig. 3 indicated that *Plat* dsRNA was more potent than *Plat* antisense RNA in targeting the reduction of endogenous *Plat* mRNA. As expected, a ten-fold dilution of *Plat* antisense RNA resulted in levels of tPA activity

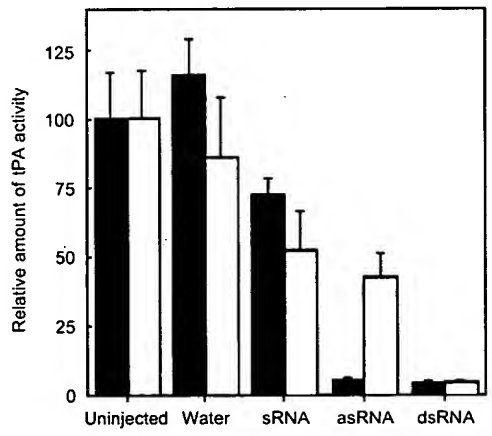

Fig. 8. Effect of *Plat* sense (s), antisense (as) and dsRNA (ds) on the appearance of tPA activity following oocyte maturation. Black bars, injection of approx. 10^6 molecules of RNA; white bars, injection of approx. 10^5 molecules of RNA. The value obtained in the uninjected oocytes was taken as 100% and the other samples are expressed relative to this amount. The data are expressed as the mean±s.e.m. and typically 5-12 eggs were assayed. The experiment was performed three times and similar results were obtained in each case; a representative experiment is shown.

similar to that of sense-injected oocytes, whereas injection of *Plat* dsRNA still promoted a dramatic inhibition (Fig. 8, white bars). Similar to the results obtained with *Mos* dsRNA, these results confirmed that *Plat* dsRNA was more potent than *Plat* antisense RNA in promoting the reduction of the endogenous *Plat* mRNA.

DISCUSSION

We have demonstrated that RNAi is an effective and efficient method to inhibit the translation of maternal mRNAs that are recruited during oocyte maturation. The reduction of the targeted mRNA, namely *Mos* and *Plat*, is specific, i.e., a nontargeted mRNA is not destroyed, and is both time- and concentration-dependent; mRNA levels can be decreased by up to 90%. In addition, dsRNA is more effective than antisense RNA. The reduction of *Mos* mRNA led to a failure in MAP kinase activation that normally accompanies oocyte maturation. A consequence of this failure is that metaphase II arrest was not maintained and that the eggs underwent parthenogenetic activation with the concomitant decrease in H1 kinase activity. Likewise, reduction of the endogenous *Plat* mRNA inhibited the production of tPA following oocyte maturation. A recent report also found that injection of *Mos* dsRNA results in parthenogenetic activation of mouse eggs (Wianny and Zernicka-Goetz, 2000); it was not shown in that study, however, that the *Mos* RNA is selectively degraded and that MAP kinase failed to activate. In the mouse, RNAi, which entails microinjection of the dsRNA, should prove far superior to antisense approaches that have been used in the past, but with variable success. It should be noted that culture of oocytes in medium containing either *Plat* or *Mos* dsRNA (2 μg/μl) does not reduce the amount of the targeted mRNA (P. S., P. S. and R. M. S., unpublished). Thus, in contrast to lower species such as *C. elegans* (Fire et al., 1998) and planaria (Sanchez-Alvarado and Newmark, 1999), in which injection of the dsRNA into the animal results in the reduction of the targeted mRNA, mouse oocytes apparently lack this uptake mechanism or, if it is present, it is very inefficient.

About 1.5×10^6 molecules of either *Plat* or *Mos* dsRNA were injected when undiluted dsRNA was used. This corresponds to an intracellular concentration of 10 nM, as the volume of an oocyte is approx. 250 pl. This concentration is similar to that required to ablate *frizzled* function in *Drosophila* embryos (Kennerdell and Carthew, 1998). In those experiments approx. 0.2 fmole of dsRNA was injected into syncytial blastoderm embryos whose volume is approx. 7.3 nl and this corresponds to approx. 25 nM. Concentrations of 10 nM dsRNA are effective in an in vitro system that supports the destruction of the targeted mRNA (Tuschl et al., 1999). Significant reduction of both *Mos* and *Plat* mRNAs are also observed when only 10,000 molecules of *Mos* or *Plat* dsRNA are injected. As the oocyte contains about 10,000 each of these transcripts (Huarte et al., 1987; Keshet et al., 1988), the reduction of the endogenous mRNA appears to be very efficient. A catalytic mechanism is possible, as in other systems the number of dsRNA molecules per cell is likely to be less than the number of endogenous transcripts. For example, in *C. elegans*, injection of 60,000 *unc*-22 dsRNA into adult animals results in the twitching phenotype in approx. 100 progeny (Fire et al.,

1998). *unc*-22 expression commences when the embryos contain about 500 cells, by which time the injected dsRNA would be diluted to only a few molecules per cell. Alternatively, the recent finding that an RNA-directed RNA polymerase is implicated in RNAi (Smardon et al., 2000) could provide an amplification mechanism that accounts for the efficacy of stoichiometric or substoichiometric amounts of dsRNA to promote the efficient reduction of the targeted mRNA.

When approx. 10^6 or 10^4 molecules of either *Mos* or *Plat* dsRNA are injected, the kinetics of *Plat* mRNA degradation are slower than that for *Mos* mRNA. As it has been estimated that oocytes contain approx. 10 000 transcripts of each of these mRNAs, the difference in kinetics of mRNA degradation may reflect that the *Mos* mRNA is more accessible to be targeted for destruction. It should be borne in mind, however, that estimate of the number of transcripts is relatively crude, and hence the difference in kinetics of mRNA degradation may reflect differences in transcript abundance, i.e., there is less *Mos* mRNA than *Plat* mRNA.

Both *Mos* and *Plat* antisense RNA are also effective in reducing the amount of endogenous *Mos* and *Plat* mRNA, respectively. Nevertheless, on a molar basis, the antisense RNA is not as effective as dsRNA. For example, *Mos* dsRNA more effectively inhibits the activation of MAP kinase when compared with *Mos* antisense RNA; parthenogenetic activation and the concomitant reduction in histone H1 kinase activity are only observed in *Mos* dsRNA-injected eggs, and not in *Mos* antisense RNA-injected eggs. This suggests in turn that MAP kinase activity must be reduced below a threshold level at which MAP kinase activity is almost absent, in order to make the eggs exit metaphase II arrest and enter interphase. Moreover, when the amount of injected *Mos* dsRNA and antisense RNA are reduced 10-fold, *Mos* dsRNA is still highly effective in inhibiting the increase in MAP kinase activity whereas *Mos* antisense RNA is essentially ineffective. Thus, *Mos* dsRNA is more efficient than *Mos* antisense RNA. A similar situation is also found with *Plat* antisense and dsRNA. Injection of 10^6 molecules of *Plat* antisense or dsRNA results in both destroying the *Plat* mRNA and inhibiting the increase in tPA activity that accompanies oocyte maturation. In contrast, injection of 10^5 molecules of *Plat* antisense RNA results in little reduction of the endogenous mRNA and little inhibition in the increase in tPA activity, while *Plat* dsRNA still results in the reduction of the endogenous mRNA and inhibition of the appearance of tPA activity.

The increased potency of dsRNA when compared with antisense RNA could, in principle, reflect differences in their stability, i.e., dsRNA is more stable than antisense RNA. This possibility is minimized by the observation that in a *Drosophila* cell lysate that supports RNAi-mediated mRNA destruction both capped antisense and capped dsRNA are stable but only the capped dsRNA is active (Tuschl et al., 1999). Moreover, results of recent experiments suggest that processing the dsRNA to discrete 20-25 nucleotide fragments is part of the mechanism that leads to destruction of the targeted mRNA (Hammond et al., 2000; Zamore et al., 2000). In fact, asRNA can give rise in an in vitro system to small amounts of stable 20-25 nucleotide fragments (Zamore et al., 2000). This could account for the activity, albeit reduced, of antisense RNA, relative to dsRNA.

dsRNAs in mammalian cells typically activate protein kinase PKR that phosphorylates and inactivates eIF2a (Fire, 1999). The ensuing inhibition of protein synthesis ultimately results in apoptosis. This sequence-independent response may reflect a form of primitive immune response, since the presence of dsRNA is a common feature of many viral lifecycles. Mouse oocytes, however, clearly lack this response, as oocyte maturation beyond germinal vesicle breakdown requires protein synthesis (Wassarman et al., 1976), which probably reflects a requirement for cyclin B synthesis, and the oocytes injected with dsRNAs resume meiosis and mature to metaphase II. Preimplantation mouse embryos also lack the response, as embryos injected with dsRNAs develop to the blastocyst stage (Wianny and Zernicka-Goetz, 2000). When the embryo acquires this response is unknown. It is not known if oocytes and preimplantation embryos contain PKR activity, which could account for the lack of the response. The lack of this response, however, cannot be attributed to a deficiency in the cell death machinery, because both oocytes (Perez et al., 1999) and preimplantation embryos (Brison and Schultz, 1997; Handyside and Hunter, 1986; Pierce et al., 1989; Weil et al., 1996) can undergo apoptosis.

The lack of this response to dsRNA may confer a selective advantage by minimizing reproductive wastage. Both oocytes and preimplantation embryos are exposed to viruses. Viral exposure throughout the lifespan of the female could deplete the pool of oocytes and compromise her reproductive capacity, because oocytes do not proliferate. The preimplantation embryo is also susceptible to viral infection from viruses present in the female reproductive tract. Infection of an early cleavage stage preimplantation embryo that results in blastomere death could result in a blastocyst containing an insufficient number of inner cell mass cells, and hence be incapable of development to term.

RNAi clearly offers several advantages to the current methods that employ generation of null mutants by homologous recombination, which requires (1) generating a suitable targeting construct, (2) selecting homologous recombination events in ES cells, (3) injecting blastocysts with these ES cells, and (4) establishing pure breeding lines from the chimeric offspring. The RNAi response will also likely be far more efficient and consistent than the antisense RNA approach that has been used with very inconsistent results in the mouse oocyte and embryo. Moreover, hypomorph phenotypes may become manifest, as RNAi does not appear to result in the total ablation of the targeted mRNA. Such hypomorph phenotypes may be as informative (or more informative) than the corresponding null mutation by providing novel insights into the presence of thresholds and/or the function of a gene. For example, as described above, a critical amount of MOS activity appears required for the development of a level of MAP kinase activity that is sufficient to maintain metaphase II arrest, a result consistent with a recently proposed switch mechanism for MAP kinase activation, as well as other cellular switches (Ferrell, 1999). In addition, modest changes in the levels of *Oct4* (*Pou5f1* – Mouse Genome Informatics) expression may also function as a developmental switch by regulating the fate of embryonic stem cells, e.g., high levels lead to differentiation into primitive endoderm and mesoderm, intermediate levels lead to pluripotent stem cells and reduced levels result in trophectoderm (Niwa et al., 2000).

As more dormant maternal mRNAs are identified, RNAi will be a valuable tool with which to study their function in oocyte maturation, fertilization and egg activation, and development. Moreover, the method can also be used to study the function of genes that are expressed in the early embryo, since dsRNA can inhibit the function of zygotically expressed genes (Wianny and Zernicka-Goetz, 2000; P. S., P. S. and R. M. S., unpublished). Whether dsRNA can also lead to DNA methylation of the targeted gene and result in long-term repression of transcription, as apparently occurs in plants (Wassenegger et al., 1994), is unknown.

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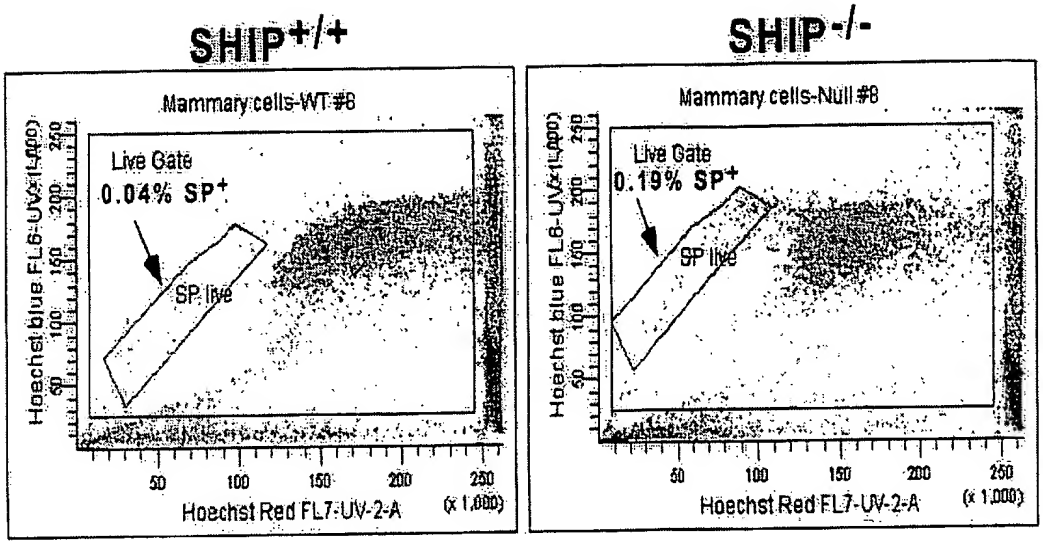
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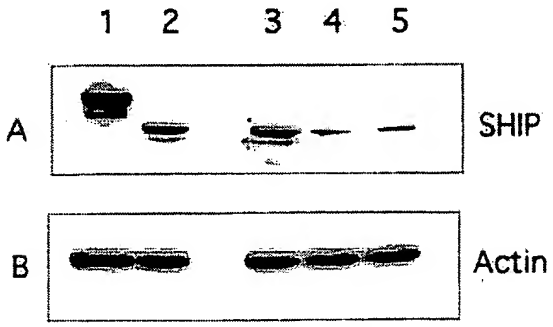
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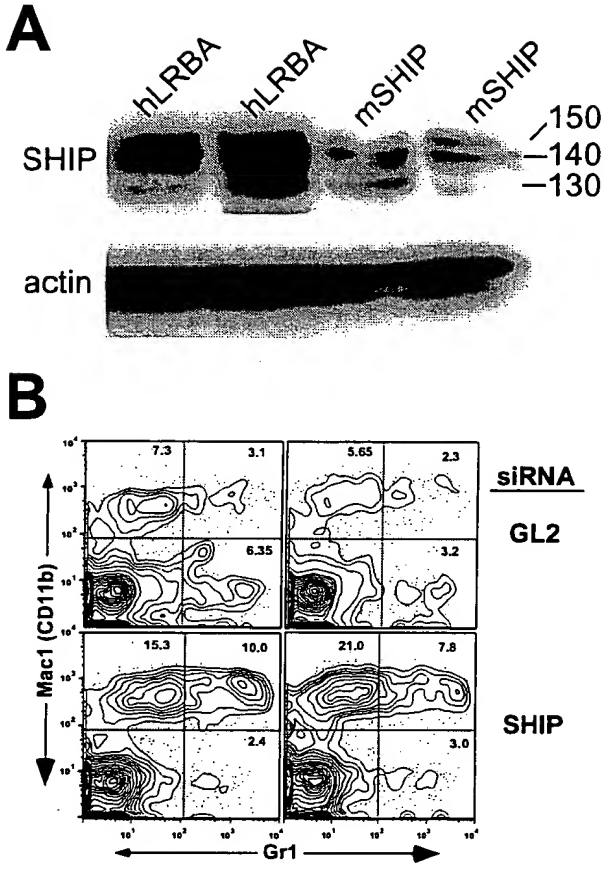
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3.0
SHIP
Mac1 (CD11b)
Gr1

muSHIP1shRNA VECTOR

- pBluescript MCS: Bam H1 and Eco R1
- mouse RNA Pol III U6 promoter
- S-SHIP TARGET: 683-704 (AF184912)
  = SHIP TARGET: 1423-1444 (MMU52044)
- Hairpin Loop=Hind III
- Pol III Transcription Termination Signal

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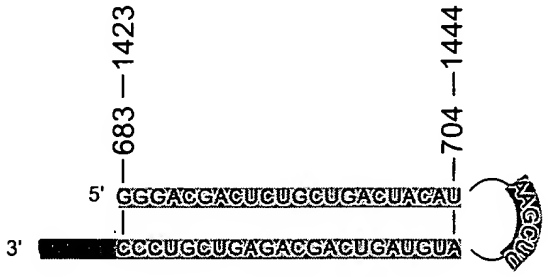

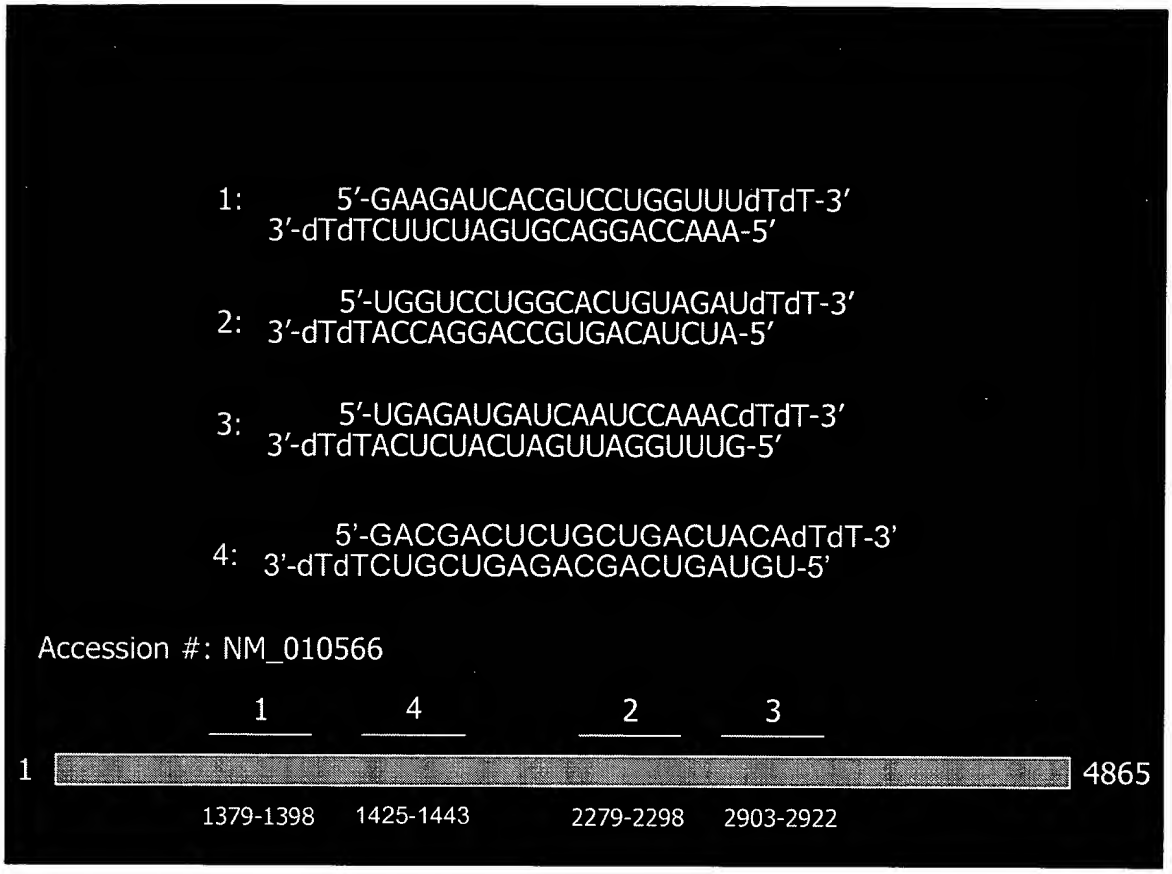
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